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**Studies on reproductive compensations of host plant
against floral herbivore**

**-Interspecific relationship between *Phengaris teleius*
and *Sanguisorba tenuifolia* var. *alba*-**

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A Doctoral Dissertation

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Summary

Biological interactions include mutualistic relationships that have a positive effect on the fitness of each other, and parasitic relationships that one species has a negative effect on the fitness of the other species. Furthermore, organisms can be classified into generalists who can relate to various organisms and specialists who can only relate to specific organisms. Plants have evolved various defense functions against feeding damage by herbivores, but specialist herbivores can adapt to their defense functions and feed on the plants. In this thesis, I investigated to clarify the reproductive strategies of the perennial herbaceous plant *Sanguisorba tenuifolia* var. *alba* against herbivory of the specialist floral herbivore *Phengaris teleius*, as well as the variation of defense functions against herbivory among years and among populations. Finally, I also examined the existence of positive contribution of *P. teleius* such as pollinator besides the floral herbivore.

Chapter 1. The defense strategies of S. tenuifolia against herbivory by P. teleius

Plants that are consumed by herbivores incur a reduction in fitness. Therefore, plants need to avoid and/or endure herbivory using ‘resistance’ and/or ‘compensation’ systems. *Phengaris teleius* is a specialist herbivore of *Sanguisorba tenuifolia*. *Phengaris teleius* lays eggs in the flower buds (i.e., spikes) of *S. tenuifolia* and the larvae of *P. teleius* feed on the ovaries and ovules of *S. tenuifolia*. In order to clarify the extent and effect of herbivory damage by *P. teleius* on reproduction in *S. tenuifolia*, I conducted field observations and artificial cutting experiments carried out in the natural habitat where both *P. teleius* and *S. tenuifolia* live sympatrically. *Phengaris teleius* often laid one egg per individual of *S. tenuifolia* and tended to lay an egg on the spikes attached to the

shoot apex (spike I). Almost all fruits were damaged in the spike I with egg. However, in between undamaged spikes (i.e., spikes other than spike I), the number of fruits of individuals with damaged spike I was larger than that of individuals with undamaged spike I. As a result, in the fruit production at the individual level, the number of fruits produced was higher in the individuals with damaged spike I. This suggested that compensatory response would be made by increasing the number of fruits in other spikes without damage. On the other hand, because no compensatory response was detected in the cutting experiments, it was considered that compensatory response may be induced by biological stimuli by *P. teleius*. From these results, the oviposition tendency of *P. teleius* and the compensatory response against herbivory may be one of the mechanisms for continuing the interspecific relationship between host plants *S. tenuifolia* and floral herbivores *P. teleius*.

Chapter 2. Seasonal and populational comparison of compensatory response of S. tenuifolia

Variations in the size and phenology of plant lead to differences in probability and timing to receive herbivory, and it has been suggested that they make differences in the results of compensatory response against herbivory. In this study, I examined whether the compensatory response of *S. tenuifolia* is a mechanism that exists consistently even in different survey years at the same population and in different populations occurring at various habitats. In interannual comparison for four years at KHa population, compensatory response was in all years. Besides KHa population, the compensatory response of *S. tenuifolia* was also detected in four populations. However, no compensatory response was observed in some populations. As a result of comparing the

individual size, flowering phenology, and fruit production of *S. tenuifolia* among populations, no association was found with or without compensatory response in factors other than fruit set. This suggests that the individual size and flowering phenology of *S. tenuifolia* do not affect the existence of compensatory response. At the population in which the compensatory response was confirmed, fruit set was potentially high in the individuals with no herbivory. This suggests that the potential abilities of fruit production of *S. tenuifolia* at each population may affect the function of compensatory response against herbivory by *P. teleius*.

Chapter 3. Effectiveness of P. teleius for fruit production of S. tenuifolia

In order to understand the effects of feeding damage on seed reproduction between herbivores and host plants, it is necessary to evaluate not only the negative effects of herbivory but also the possible contribution to seed reproduction. *Phengaris teleius* adults have often been observed to visit flowers of *S. tenuifolia*. In this study, I focused on the function of *P. teleius* as the pollinator to *S. tenuifolia*. *P. teleius* was a major flower visitor of *S. tenuifolia* from early August to mid- August (i.e., the early to mid-flowering period), but thereafter ants became major flower visitors. It was confirmed that the scales of *P. teleius* that visited *S. tenuifolia* had pollens attached to them, and that pollens were transferred from *S. tenuifolia* to *P. teleius*. By the investigations using fluorescent powder as pseudo pollen, it was observed that the powder was attached to the stigma of *S. tenuifolia*. However, regardless of the presence or absence of *P. teleius* visit, there was no difference in fruit set of *S. tenuifolia*. From these facts, it is considered that *P. teleius* visit is unlikely that it will contribute to fruit production of *S. tenuifolia*.

Conclusion

The series of studies have shown that the host plant *S. tenuifolia* maintains fruit production through compensatory response to the herbivory of the specialist floral herbivore *P. teleius*. Compensatory response had been confirmed in multiple years at the same population and various populations, it suggested that the compensation response of *S. tenuifolia* against herbivory is an evolutionarily stable mechanism. In addition, since no compensatory response was confirmed in the population with a low fruit set even in individuals without herbivory, the fruit production capacity in that environment may affect the existence of compensatory response. The results obtained in these studies provided important insights into the defense mechanism of plants against feeding damage by specialist herbivores. In addition, elucidation of the reproductive compensative function of plants against floral herbivores is considered to be very meaningful in considering the population maintenance of both species and the sustainability and conservation of the ecosystem.

General introduction

Organisms survive and reproduce interacting with other organisms and form an ecosystem. Biological interactions (both direct and indirect) occur between species or individuals, and the effect of interactions among species are various, i.e., positive, neutral, or negative (Bardgett & Wardle 2003). A relationship in which two species have a positive effect on fitness is called a mutualistic relationship. Well-known mutualistic relationships are the relationship between legumes and rhizobia (Faria *et al.* 1989) and the relationship between ants and aphids (Kaplan & Eubanks 2005). On the other hand, the relationship in which one species has a negative effect on fitness of the other species is called a parasitic relationship. In parasitic relationships, the host species may be killed or be adversely affected the growth and/or reproduction of host species (Karowe & Schoonhoven 1992; Glatzel & Geils 2009).

These interspecific relationships can be classified into two categories. One is ‘generalists’ who can relate to a variety of organisms and the other is ‘specialists’ who can only relate to specific organisms. For example, plant-eating herbivores include generalists who can feed on a variety of plant species such as sheep and goats (Kam *et al.* 2012), and specialists who feed only on specific species such as panda (bamboo eater) and koala (eucalyptus eater) (Wilson & Hayek 2015). There are many specialists in herbivorous insects that have been co-evolved with plants. For example, lepidopteran larvae usually feed on only a few species of plants belonging to one family (oligophagous) or only one genus (monophagous) (Sielezniew & Stankiewicz 2013). Specialists who feed only on specific plant species are more vulnerable to sudden changes in the environment than generalists who can feed on a variety of plant species (Shipley *et al.* 2009). Since the specialist cannot survive in the absence of a host

species, host species population maintenance is important for the conservation of specialists.

Host organisms have evolved various defense functions to escape the negative effects such as feeding damage. For example, a strategy that mimics the surrounding background to make it difficult for predators to find (e.g., phasmids, Bedford 1978) and a strategy that avoids prey by pretending to be dead (thanatosis; e.g., red flour beetles, Humphreys & Ruxton 2018). Since plants cannot move and escape from herbivores, they also avoid feeding by mechanical resistance such as thorns and trichomes (Levin 1973) and chemical resistance such as secondary metabolites (Whittaker & Feeny 1971) (Fig. 1-1). However, specialist herbivores can adapt to their defense mechanisms and feed on the plants (e.g., cabbage butterflies, Vergara *et al.* 2006; koalas, Shiffman *et al.* 2017). Understanding adaptation strategies in plants that defense functions do not work (or do not have defense functions themselves) against the feeding damage of specialist herbivores is important for further understanding of biological interactions.

The scarce large blue *Phengaris (Maculinea) teleius* (Lycaenidae) is a specialist herbivore of *Sanguisorba* (Rosaceae) plants (Thomas 1984; Suda *et al.* 2012).

Phengaris teleius is widely distributed in Asia, Europe, and Russia (Wynhoff 1998; Yada 2007; Suda *et al.* 2012). However, this butterfly is endangered around the world, including in Japan (van Swaay & Warren 1999; Ministry of the Environment, 2015). Adult females lay eggs on buds of *Sanguisorba* plants (Fig. 1-2a), and hatched larvae feed on ovaries and ovules in the spike until the larvae become a last instar (fourth instar; Fig. 1-2b). *Phengaris teleius* adult survives for some time after oviposition and may visit the *Sanguisorba* flowers. After that, the larvae drop to the ground and are taken by the workers of *Myrmica* ants to their nest (Thomas 1984; Thomas & Elmes

1998). And, they feed on ant brood until summer one or two years later.

The genus *Sanguisorba* (Rosaceae) is distributed in the northern hemisphere, including Asia, Europe and North America (Satake *et al.* 1982). In Japan, seven species of *Sanguisorba* are known to distribute, and *S. tenuifolia* Fisch. ex Link var. *alba* Trautv. et Mey. is distributed from Honshu and Hokkaido (northern part of Japan) in Japan and mainly grows in grasslands and wetlands (Satake *et al.* 1982). Flowering individuals have multiple long flowering spikes consisting of small white florets (Fig. 1-2c). The florets have no petals, and the four white sepals look like petals. There are four stamens protruding from the center per floret. The pistil is located in the center of the florets and the stigma is lower than the stamens. In Hokkaido, the spikes are laid eggs by *P. teleius* from late July to early August (Nagamori *et al.* 2016). Flowering starts in mid- August and each flower in a spike produces one fruit (and one seed) per flower (Fig. 1-2d).

If the flowers of *Sanguisorba* plants are unilaterally eaten by *P. teleius*, it is considered to have a direct negative effect on the seed reproduction. If *Sanguisorba* plants are not produced seeds due to herbivory by *P. teleius*, it is considered that the maintenance of *Sanguisorba* population will also be negatively affected. Nevertheless, the interspecific relationship with *P. teleius* has been maintained for many years (Thomas *et al.* 2004). From this, it is expected that (1) *Sanguisorba* plants have defense strategies that is not resistance traits, and/or (2) *P. teleius* contributes to seed production of *Sanguisorba* plants. In this study, I will understand the reproductive strategies of host plants against feeding damage of floral herbivores through the interspecific system of *P. teleius* and *Sanguisorba tenuifolia* var. *alba*. First, in Chapter 1, I clarified the defense strategies of *S. tenuifolia* against the herbivory of *P. teleius* by confirming the actual

condition of feeding damage by *P. teleius* and the fruit production of *S. tenuifolia*. In Chapter 2, I examined the variation about defense strategies of *S. tenuifolia* by conducting verification in multiple *S. tenuifolia*- *P. teleius* populations. In Chapter 3, I evaluated the effectiveness of *P. teleius* on fruit production of *S. tenuifolia* by confirming whether *P. teleius* behaves not only a herbivore but also a pollinator.

Chapter 1. The defense strategies of *S. tenuifolia* against herbivory by *P. teleius*

1.1 Introduction

Plant organs such as leaves, stems, flowers and fruits, are consumed by various herbivores. Consumption by herbivores causes various damages to plants and possibly reduces the fitness of individual plants (Marquis 1984). Defense strategies against herbivory basically consist of two major systems (Strauss & Agrawal 1999; Stowe *et al.* 2000; Núñez-Farfán *et al.* 2007) (Fig. 1-1). One is ‘resistance’ (Karban & Baldwin 1997), which can be divided in three types, i.e. mechanical resistance (e.g., Levin 1973), chemical resistance (e.g., Whittaker & Feeny 1971) and biological resistance (e.g., Dicke & Sabelis 1987; Heil *et al.* 2004). Mechanical resistance consists of physical defenses to prevent herbivores, including morphological traits such as spines or trichomes on the leaves and branches (Levin 1973). Chemical resistance in plants involves the production and accumulation of secondary metabolites that may kill herbivores or prevent digestion by herbivores (Whittaker & Feeny 1971). Biological resistance includes attraction of ants or carnivorous insects using extrafloral nectary or volatile organic compounds (Dicke & Sabelis 1987; Heil *et al.* 2004). The other methods ‘compensation’, which compensates for the fitness reduction inflicted by herbivory by regrowing vegetative tissues such as leaves and stems, increasing resources put into reproduction, and increasing photosynthetic rates (Strauss & Agrawal 1999; Stowe *et al.* 2000; Fornoni 2011). Compensatory response sometimes result in overcompensation in which plant individuals affected by feeding are more fitness than individuals not affected by feeding (Huhta *et al.* 2000; Stowe *et al.* 2000).

Until the 1990s, overcompensation induced by vertebrate herbivory was

reported (e.g., Paige 1999), while research on overcompensation against insect herbivory had not progressed (Agrawal 2000). This is because insect herbivores generally consume less plant biomass than vertebrate herbivores and were not considered necessary to compensate (Kotanen & Rosenthal 2000). However, in recent years, there are many studies on overcompensation to herbivory by insects. Agrawal and Fishbein (2008) reported an increase in regrowth capacity of *Asclepias* plants against herbivory by insects. Wise and Abrahamson (2013) reported an increase in seed production of *Solidago altissima* (Asteraceae) against herbivory to *Philaenus spumarius* (Cercopidae). A meta-analysis by Garcia and Eubanks (2019) revealed 86 studies documenting examples of overcompensation for insect herbivory by 67 plant species representing 26 families. This may indicate that overcompensation for insect herbivores damage is common for plants.

Sanguisorba tenuifolia var. *alba* (Rosaceae) is a perennial herb and is known to be a food source to the larvae of *P. teleius* (Lepidoptera: Lycaenidae) (Thomas 1984; Suda *et al.* 2012). *Sanguisorba tenuifolia* produces multiple spikes (inflorescences with flowers arranged on the axis) from one individual, and the ovaries and ovules are damaged by *P. teleius*. Therefore, it is reasonable to consider that feeding pressure of *P. teleius* has negative effects on reproduction, especially fruit and seed production of *S. tenuifolia*. Since no resistance mechanism against flower feeding by *P. teleius* was confirmed in *S. tenuifolia*, it was thought that there might be strategies to prevent the decrease in seed production by causing a compensatory response against feeding damage.

1.2 Materials and methods

Study site

Field observations and experiments were conducted in an open grassland (area 1,500 m²) in Kitahiroshima City, Hokkaido, Japan, from 2015 to 2016 (Table 1-1, Fig. 1-3; KHa population). This site has been conserved for *P. teleius* for many years. This site is regularly mowed in early June every year before the flower stems of *S. tenuifolia* start to grow. In addition, in order to prevent the overgrowth of a tall grass *Miscanthus sinensis*, *M. sinensis* were often cut as appropriate. At this site, both *S. tenuifolia* and *P. teleius* live sympatrically.

Characteristics of each spike of S. tenuifolia

Phengaris teleius lays eggs on the spikes of *S. tenuifolia*. Because *S. tenuifolia* produces multiple spikes per individual, I investigated the formation patterns of the spikes and the characteristics of each spike. I marked 20 plants and periodically monitored spikes in 2015. In these observations, in order to confirm the essential characteristics of the spikes, the marked plants were entirely covered with a net (200 cm in height and 16 mm mesh size) to prevent oviposition and herbivory by *P. teleius*. The positions of the spikes of each individual were numbered as I, II, III, etc., in order from the spike attached to the shoot apex (Fig. 1-4). I counted the number of spikes on marked plants and measured various characteristics such as the length of each spike, the number of fruits, fruit set per spike and dry weight of fruits. Fruit set was calculated as the fruit/flower ratio; the ratio of the total number of fruits produced to the total number of flowers per spike.

Effect of oviposition and herbivory by P. teleius

First, I counted the number of *S. tenuifolia* individuals with flower stem that indicated *P. teleius* may lay eggs at KHa population. For those *S. tenuifolia* individuals with flower stems, I observed the oviposition of *P. teleius* about three times a week and marked those individuals that were found with eggs or larvae on the spike, from late July to mid- August in 2016. The marked plants had the height of flower stem measured, and the position of the spike with eggs and the number of laid eggs were recorded.

I randomly selected 45 *S. tenuifolia* individuals in mid- July. After fruiting (after September), the spikes were collected and were measured the number of fruits, fruit set per spike and dry weight of fruits. Fruit set was calculated as the ratio of the total number of fruits produced to the total number of flowers per spike. The dry weight of fruits was measured using only undamaged fruits.

With counting the number of fruits, I checked for feeding damage by *P. teleius*. On the fruits damaged by *P. teleius*, there are feeding scars at the bottom of fruits. Feeding damage rate per spikes of *S. tenuifolia* by *P. teleius* was assessed by the number of fruits damaged per total number of fruits produced in the spike. Feeding damage rate per individual was assessed as the ratio of the number of damaged spikes to the total number of spikes per individual. Feeding damage rate per population was assessed as the damaged individual/total observed individual ratio; the ratio of the number of individuals with at least one damaged spike to the total number of observed individuals.

Test of compensatory response by cutting experiments

Cutting experiments as imitative herbivory have been used effectively to test defense and compensatory response of plants (Marquis 1992; Ishizaki *et al.* 2010). In 2015, I

randomly selected 50 individuals of *S. tenuifolia* at this study site in mid- July. I performed cutting experiments on the spike at the shoot apex (spike I) at two different stages of spike development, Cut-1 and Cut-2 (Fig. 1-5): Cut-1: spike I was cut when the spike started to appear at late July; Cut-2: spike I was cut when the flowers began blooming in mid- August. The timing of Cut-2 was the same period as herbivory by *P. teleius* starts. 25 individuals from 50 selected individuals were used for each treatment. I also randomly marked 20 individuals of *S. tenuifolia* that were not cut as a control in mid- July. To avoid oviposition and herbivory by *P. teleius*, the control plants were entirely covered with a net. For all individuals in the cutting experiments, I performed weekly measurements of the height of flower stem, the number of spikes and the length of each spike from late July to early November. After that, I collected the fruiting spikes and measured the number of fruits, fruit set per spike and dry weight of fruits.

Because *S. tenuifolia* is a perennial plant, the effect of cutting, i.e., herbivory damage, may appear in the subsequent year, I randomly chose individuals from the marked plants, specifically seven of 25 individuals in 2015. In 2016, those seven individuals were entirely covered with a net to avoid herbivory by *P. teleius*. Then, as in 2015, I measured the height of the scape, number of spikes, length of each spike, number of fruits, fruit set per spike and dry weight of fruits.

Statistical analysis

T-test was used to compare the number of fruits and fruit set between spikes (or individuals) damaged by herbivory and those not damaged. GLMM was used to analyze the number of spikes, the number of fruits (Poisson distribution) and fruit set (binomial distribution) and LMM was used to analyze the length of spikes and dry weight of fruit.

These tests were used for the comparison among spikes and the results of cutting experiment. Also, Tukey's test was used to compare the differences among each spike or among treatments of cutting experiment. Each character (number of spikes, length of spikes, number of fruits, fruit set, and dry weight of fruits) was analyzed using LMM or GLMM with spike number (I, II, III, ...) or treatments (control, Cut-1 or Cut-2) as independent variables, each item as the dependent variable, and individuals as a random effect, followed by a Tukey's test. To compare the number of fruits for each the feeding damage rate per individuals, I analyzed using GLMM (Poisson distribution) with feeding damage rate (at 20% intervals) as an independent variable, the average number of fruits per spike as the dependent variable, and individuals as a random effect, followed by a Tukey's test. To compare the performances of the same individual between 2015 and 2016, each item was analyzed using paired *T*-test. LMM or GLMM were carried out with R 3.5.2 using library 'lmer' or 'glmer' function in the lme4 and Tukey's tests were run with 'ghlt' function in the multcomp packages (R Core Team 2018).

1.3 Results

*Characteristics of each spike of *S. tenuifolia**

The number of spikes per individual varied from three to nine with an average of 6.5 ± 0.4 (mean \pm SE). Then, I compared the characteristics from spike I to VII which were produced more than half of marked individuals. For the length of spikes, the spike at the shoot apex (spike I) was 62.08 ± 4.69 mm and this spike was larger than the other spikes (Table 1-2). Additionally, after spike II, the spikes close to spike I (II, III, IV, V) were smaller, but the spikes attached to the latter half (VI, VII) were found to be about

the same size as spike I (Tukey's test, $P < 0.05$). There was a positive correlation between the length of a spike and number of flowers ($N = 70$, $R^2 = 0.73$, $P < 0.05$). With regard to the number of fruits, although there was a significant difference between several spikes (ex. spike I vs spike II, Tukey's test, $P < 0.05$), the number of fruits was almost the same among all spikes (e.g., spike I vs spike IV, Tukey's test, $P = 0.61$). Similarly, although there was a significant difference between several spikes (e.g., spike I vs spike V, Tukey's test, $P < 0.05$), the fruit set was almost the same among all spikes (e.g., spike I vs spike IV, Tukey's test, $P = 0.19$). The dry weight of fruits was not significantly different between the spikes at the different position (Tukey's test, $P > 0.17$).

*Oviposition place and number of eggs of *P. teleius**

In total, 1146 individuals of *S. tenuifolia* were marked and monitored at KHa population in 2016. Of 1146 individuals, 269 had eggs laid by *P. teleius*. In addition, of 269 individuals, 61% (164 individuals) had only one egg laid (number of eggs per individual = 1-10, mean = 1.78 ± 0.09). Furthermore, 55% (299 eggs) of all eggs were laid in spike I.

Degree of feeding damage and effect of fruit production

In 2016, the 168 spikes that had been eaten were observed, and the feeding damage rate ranged from 31 to 100%, with an average of $82.9 \pm 1.7\%$ (mean \pm standard error; Fig. 1-6). About half of the damaged spikes (82 spikes) were eaten on more than 80% of the fruits. The average number of fruits in damaged spike I was 21.7 ± 4.3 fruits, and the average fruit set was $14.7 \pm 2.7\%$, which were significantly lower than those of the

undamaged spikes (60.1 ± 13.5 fruits, $35.8 \pm 7.2\%$ respectively; Fig. 1-7a).

On the other hand, the number of flowers and fruits were compared between the spikes that were not damaged (spike II-V). As a result, although the number of flowers did not increase, the number of fruits produced by individuals damaged on the spike I was larger than that of the individuals undamaged (Fig. 1-7b). The number of flowers was higher in the damaged individuals only in spike III, but that did not change in the other spikes. In spike II and spike III, the number of fruits produced by individuals damaged on the spike I more than doubled compared to individuals undamaged. There was no statistically significant difference in spike IV, but individuals damaged on the spike I tended to have more fruits compared to individuals undamaged. In spike V, the number of fruits increased in the individuals damaged. As a result of the increase in the number of fruits produced in the spikes other than spike I, the average number of fruits in the all spikes was higher in individuals that were damaged on the spike I than individuals undamaged (Fig. 1-7c).

On the other hand, since multiple eggs are occasionally laid in *S. tenuifolia*, I also compared the number of fruits when multiple spikes were damaged (Fig. 1-8). As a result, the average number of fruits per spike decreased when more than 60% of the spikes were damaged (Tukey's test, $P < 0.05$).

Test of compensatory response by cutting experiments

Figure 1-9 shows the number of spikes (a), length of spike II (b), total number of fruits (c), and average dry weight of fruits (d) for each treatment (control, Cut-1 and Cut-2) in 2015. As for the number of spikes, spike I was excised in Cut-1 and Cut-2, so one was added to the number of spikes in those treated individuals for comparison. There were

no significant differences in the number of spikes between the cutting treatments (Tukey's test, $P > 0.34$). Figure 1-9 (b) shows only the results of spike II, but there were no differences among the treatments for any spike length (Tukey's test, $P > 0.85$). In terms of the total number of fruits produced, plants in the Cut-1 treatment had significantly more fruits compared with the control, whereas Cut-2 treatment, which was assumed to correspond to herbivory by *P. teleius* significantly decreased fruit number compared with the control (Tukey's test, $P < 0.05$). Although the weight of fruits was similar between the control and Cut-1 treatments (Tukey's test, $P = 0.86$), Cut-2 showed significantly decreased fruit weight compared with the control (Tukey's test, $P = 0.02$).

There were no significant differences between years (2015 and 2016) in any measurement item or in any treatment (*T*-test, $P > 0.07$). In 2016, the number of spikes and the length of spikes were similar among treatments (Tukey's test, $P > 0.52$). As for the number of fruits, only Cut-1 significantly increased than control (Tukey's test, $P < 0.05$). The weight of fruits was no difference among treatments in 2016 (Tukey's test, $P > 0.84$).

1.4 Discussion

Under natural conditions, *P. teleius* tended to lay one egg on the spike I of *S. tenuifolia*. On the spike I, almost all fruits were eaten by *P. teleius* larvae, and consequently the number of fruits and fruit set per spike decreased. However, the number of fruits of the spikes other than spike I, which was not damaged by feeding increased, and as a result, the number of fruits per individual increased compared to the individuals without feeding damage.

Phengaris teleius tended to lay eggs on spike of the shoot apex, i.e., spike I. At the end of July, when *P. teleius* begins to lay eggs, the flower stems of *S. tenuifolia* are still growing and the spikes other than spike I are still small buds. Therefore, it seems that *P. teleius* will primarily lay eggs on spike I, which is relatively large compared with other spikes. It is known that other butterflies of the genus *Phengaris* often lay eggs on large buds (Fürst & Nash 2010). In addition, since females of *P. teleius* prefer young spike without open flowers and a high percentage of flowers still enclosed by green sepals (Thomas & Elmes 2001; Wynhoff & van Langevelde 2017), it is considered that after flowering of spike I, they laid eggs on the spikes other than spike I that have not yet bloomed.

It was revealed that *P. teleius* tended to lay one egg on *S. tenuifolia* individual. This is consistent with the previous research reporting that *Phengaris* females lay only a few eggs on each plant (Thomas & Elms 2001), possibly to reduce competition for food among larvae inside the host plant (Thomas & Wardlaw 1995). In addition, the limited number of eggs laid suggests the presence of oviposition-detering pheromone (ODP) in *P. teleius* (Sielezniew & Stankiewicz 2013). However, Sielezniew and Stankiewicz (2013) showed that due to the lack of suitable spikes for oviposition, it was difficult for *P. teleius* to avoid eggs of the same species and it tended to evenly distribute the eggs among the available spikes. From this, there is a possibility that multiple eggs are laid on multiple spikes due to a decrease in the number of *S. tenuifolia* or a deviation in flowering phenology.

In this study, it was clarified that the number of fruits produced per individual of *S. tenuifolia* was almost the same or increased as that of an individual without feeding damage if only the spike I was eaten by *P. teleius*. This is thought to be due to the

reallocation of resources from spikes I to other spikes (Strauss & Agrawal 1999). Fruit set of each spike was about 30 to 40% even when there was no feeding damage. When the spike I was damaged, even though the number of flowers in the intact spikes did not increase, the number of fruits increased compared to individuals with undamaged spike I. From this, it is considered that fruits that were not matured in the absence of herbivory were newly matured to compensate for the decrease in the number of fruits due to herbivory. On the other hand, when multiple spikes were damaged, the number of fruits decreased even for the remaining spikes. This is similar results to previous studies demonstrating that moderately damaged plants compensate for negative effects on growth and reproduction (Blue *et al.* 2015). Since *S. tenuifolia* is a perennial plant, it is possible that when high feeding pressure is applied, it will refrain from breeding this year and carry over resources to the next year or later. Similar results have been obtained from studies with *Pimpinella* plants (Huhta *et al.* 2009). From the oviposition tendency of *P. teleius*, the number of damaged spikes per individual is one or two, so it is basically considered that the feeding pressure is within the range which compensatory response occurs.

The artificial cutting experiments of spike I at different times identified different response features of *S. tenuifolia*. The number of fruits increased with Cut-1 compared with the control, but decreased with Cut-2 (Fig. 1-9c), suggesting that compensatory response in the number of fruits was not observed with Cut-2. Although Cut-2 was performed just before the flowers bloomed, which was assumed to mirror the period of herbivory by *P. teleius*, the result of Cut-2 did not coincide with the results from the individuals with herbivory (Fig. 1-7, 1-9). In this case, artificial damage may have been unable to reproduce the plant response to equivalent levels of natural herbivory damage

(Heil 2010). For example, it is known that plants are able to detect and respond to biological stimuli produced by herbivores before and/or after the herbivory (Mithöfer & Boland 2008; War *et al.* 2018). Even if both real herbivory by *P. teiuis* and artificial damage of Cut-2 caused the quantitatively same damage to *S. tenuifolia*, they were qualitatively different. Therefore, it may be considered that the mechanism to compensate with seed production was induced by chemical components of the larvae of *P. teiuis* (e.g. saliva). In addition, the number of fruits in 2015 with Cut-1 was significantly higher compared with the control in 2015 (Fig. 1-9c). One possible explanation was that Cut-1 was made at an early stage of the growth phase, so compensation was possible. This means that there is a possibility that the compensation mechanisms may be different between herbivory by *P. teiuis* and damage at an early stage.

Specialist herbivores usually exhibit lower sensitivity to plant secondary metabolites compared with generalists (Ali & Agrawal 2012). Because the effect of resistance decreases for herbivores adapted to a host plant, the degree of damage to plants is expanded by increasing the consumption per herbivore and/or the population size of herbivores (Jokela *et al.* 2000; Futuyma & Agrawal 2009). Therefore, I considered that ‘compensation’ as a plant defense strategy is more adaptive against specialist herbivores, as Agrawal & Fishbein (2008) and Bustos-Segura *et al.* (2014) suggested in other plant groups. In particular, because herbivory by *P. teiuis* has a direct effect on seed reproduction of *S. tenuifolia*, the mechanism to compensate with seed production is important. Because *P. teiuis* usually laid one egg per individual plant, herbivory did not reduce reproductive potential in the year damage occurred through a compensation mechanism. These responses may be part of the mechanism for

maintenance of the interspecific relationship between *S. tenuifolia* (host plant) and *P. teleius* (herbivorous insect).

Table 1-1 Information on the study sites. The left side of "Locality" is the name of the study site, and the right side is the abbreviation. "Observation year" indicates the year investigated in a circle. "*P. terrius*" indicates whether or not *P. terrius* inhabit. The location of each study site is shown in Fig. 1.

Locality	Environments	Altitude (m)	Area (km ²)	Observation year				<i>P. teleius</i>	
				2016	2017	2018	2019		
Kitahiroshima_A	KHa	grassland	54	1.20	○	○	○	○	Present
Kitahiroshima_B	KHb	grassland	85	5.34			○	○	Present
Sapporo	SP	wetland	5	5.43			○		Present
Ishikari	IK	wetland	4	15.92			○		Absent
Bibai	BB	roadside	70	0.93				○	Present
Tomakomai_A	TKa	lakeside	4	0.74				○	Absent
Tomakomai_B	TKb	roadside	16	5.38				○	Present
Tomakomai_C	TKc	roadside	11	2.11				○	Present
Shiraoi	SO	wetland	3	6.25			○		Present
Hakodate	HD	mountain	213	6.34			○		Present
Kaminokuni	KK	mountain	140	6.58		○	○		Present

Table 1-2 Characteristics of the spikes of *S. tenuifolia*. Labels I to VII indicate the positions of the spikes of each individual starting from the spike attached to the shoot apex. Letters beside each value show the results of the Tukey's test. Same letters show no significance among the values ($P<0.05$).

Spike position	Spike length (mm)	No. of fruits	Fruit set	Dry weight of fruits (mg)
I	62.08 ± 4.69 a	43.10 ± 8.22 a	0.34 ± 0.05 ab	1.77 ± 0.12 a
II	30.09 ± 2.42 d	21.50 ± 5.17 b	0.32 ± 0.06 ab	1.69 ± 0.14 a
III	40.56 ± 2.32 c	26.25 ± 4.50 b	0.29 ± 0.04 a	1.70 ± 0.13 a
IV	47.57 ± 3.72 bc	41.75 ± 7.33 a	0.37 ± 0.05 bc	1.73 ± 0.11 a
V	51.96 ± 4.36 b	47.94 ± 9.24 a	0.40 ± 0.07 c	1.92 ± 0.10 a
VI	59.38 ± 4.09 ab	50.79 ± 9.82 a	0.42 ± 0.06 bc	1.83 ± 0.14 a
VII	59.06 ± 6.26 ab	51.92 ± 11.02 a	0.43 ± 0.08 bc	1.74 ± 0.09 a

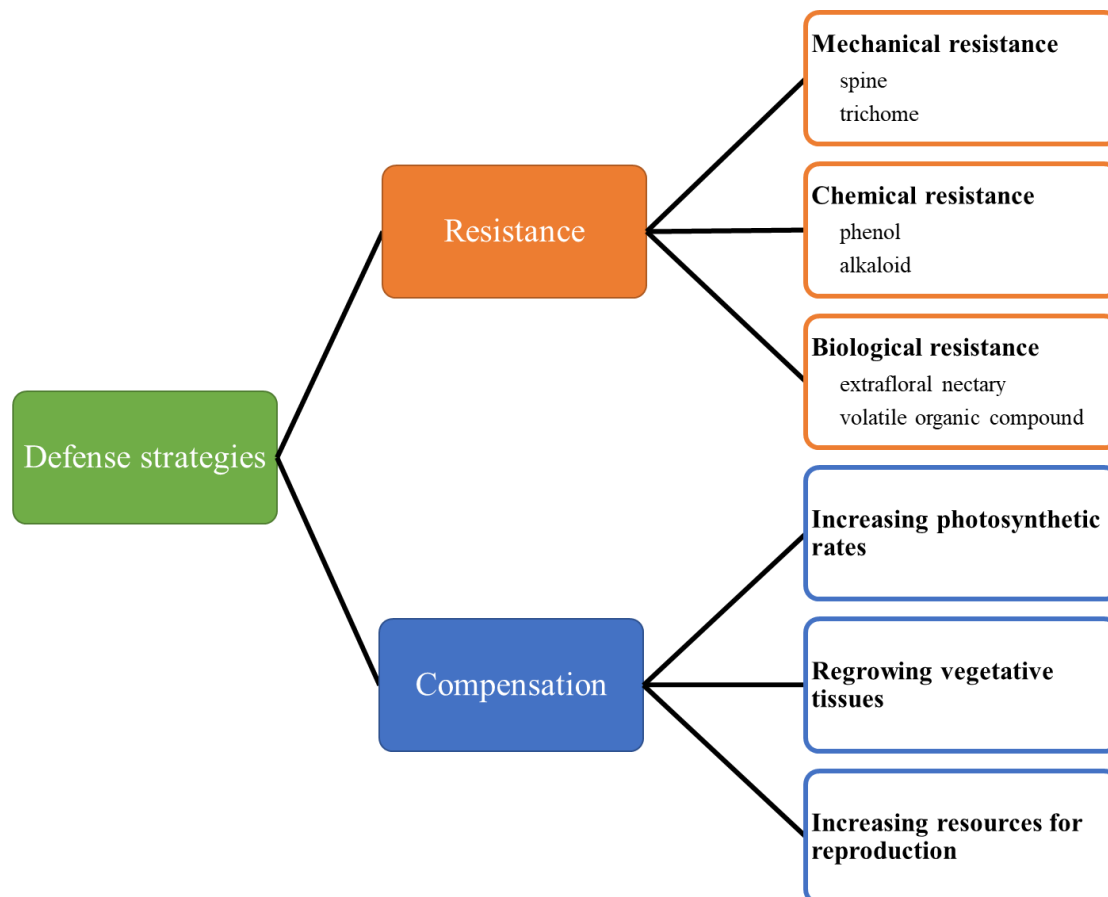


Fig. 1-1 Defense strategies against herbivory basically consist of two major systems (Strauss & Agrawal 1999; Stowe *et al.* 2000; Núñez-Farfán *et al.* 2007). ‘Resistance mechanism’ is a strategy that avoids herbivory using such as spines or secondary metabolites or carnivorous insects. ‘Compensatory response’ is a strategy that compensates for the decline in growth and seed production after feeding.

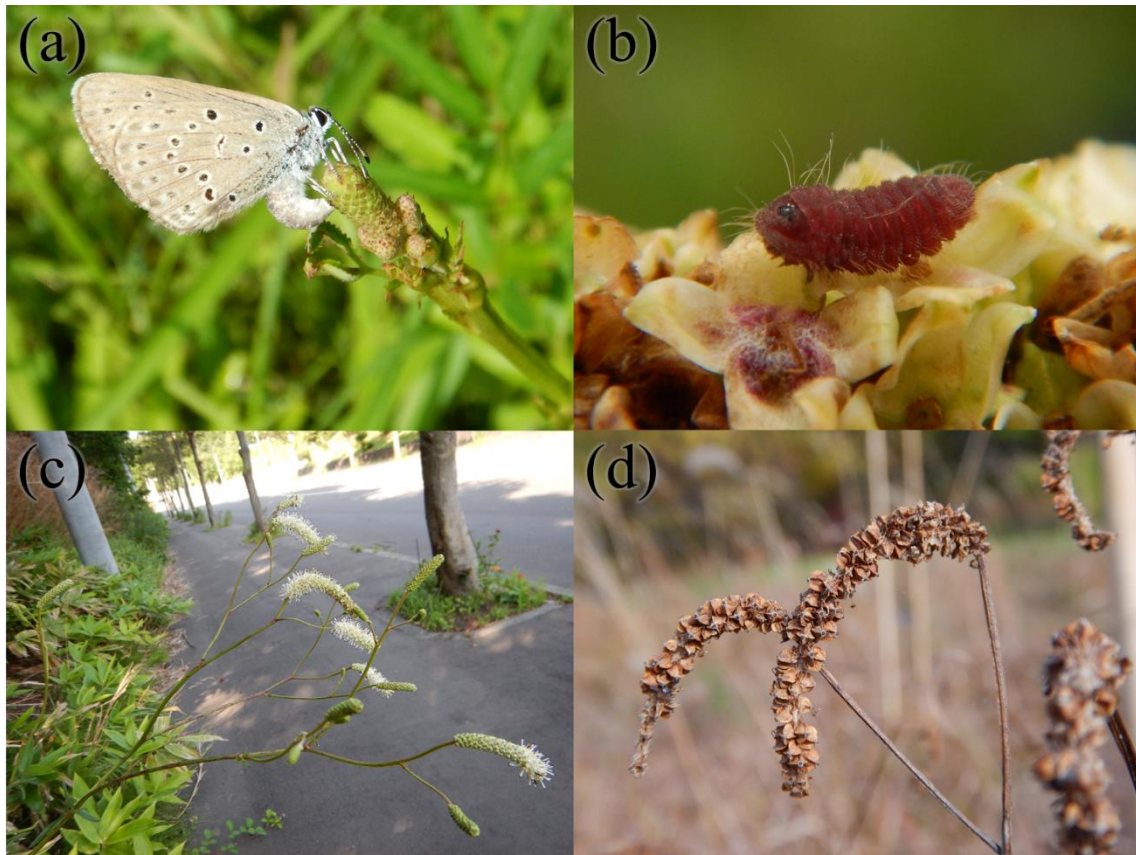


Fig. 1-2 Photographs of studied species. *Phengaris teleius* adult (a) and last instar larva (b). Adult female lays eggs to the bud of *Sanguisorba tenuifolia*. Flowering spikes (c) and matured fruits (d) of *S. tenuifolia*. Multiple fruits are produced from one spike.

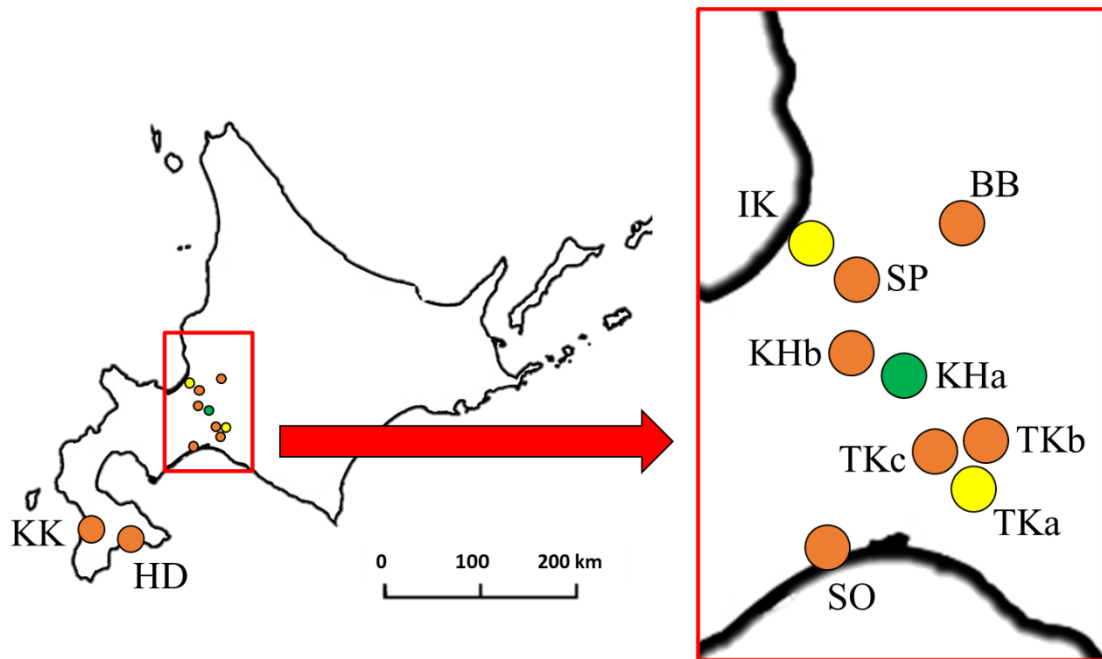


Fig. 1-3 Map showing 11 populations of *S. tenuifolia* were used observations. At the population of green plot (KHa), I conducted research about interannual comparison (Chapter 1) and pollination (Chapter 3). At the populations of orange and yellow plots, I conducted research about population comparison (Chapter 2) and *P. teleius* did not inhabit in yellow plot populations. Name and environment of each study population are shown in Table 1.

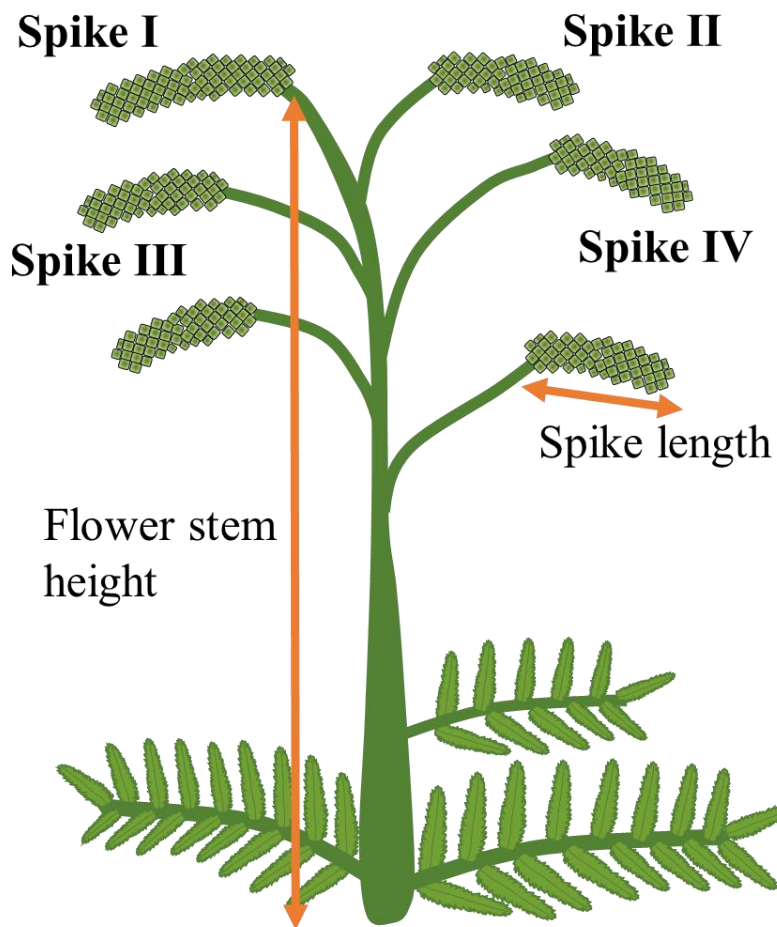


Fig. 1-4 Schematic diagram of *Sanguisorba tenuifolia*. The positions of spike of individual were numbered as I, II, III, etc., in order from the spike attached to a shoot apex. Flower stem height was the height from the ground to the base of spike I. Spike length was the length from the root to the tip of each spike.

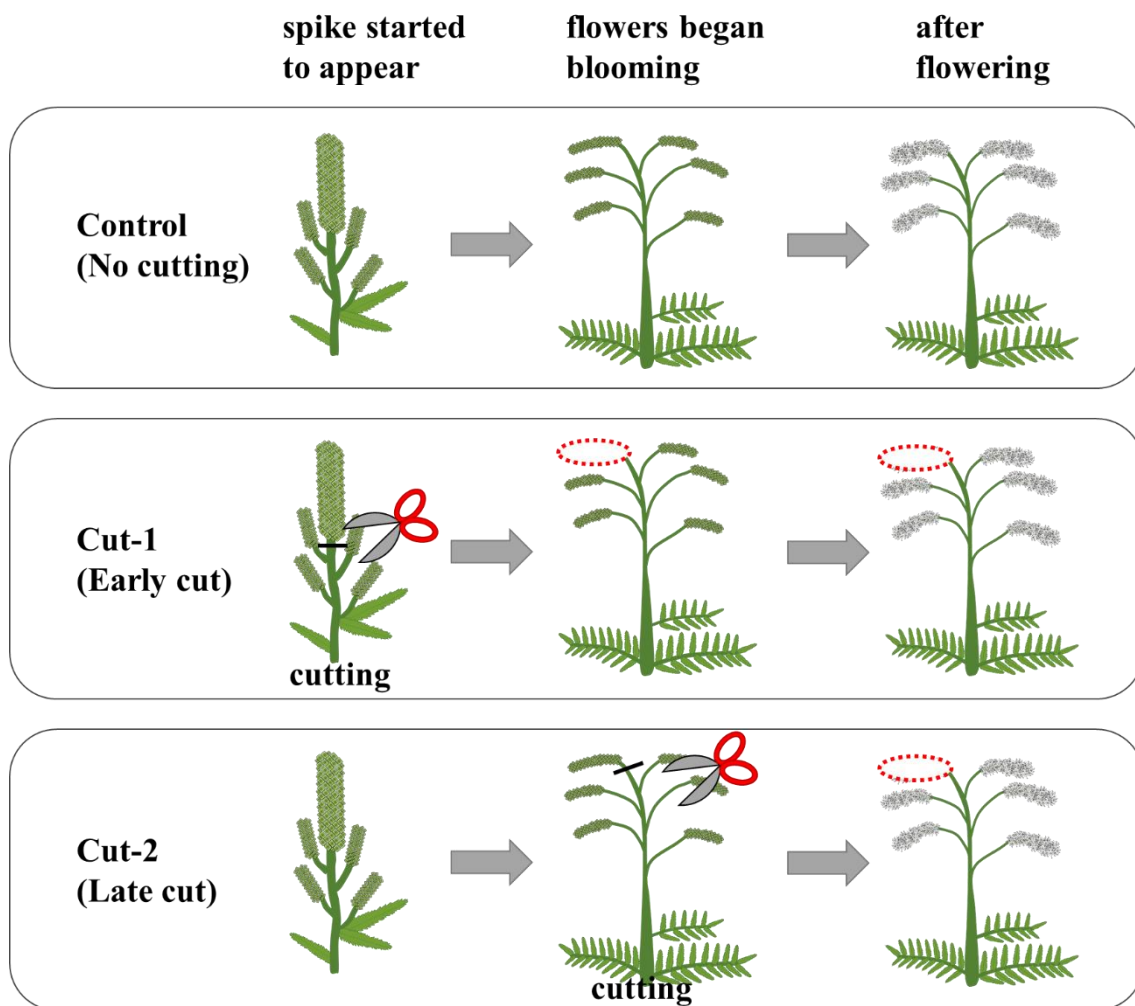


Fig. 1-5 Three treatments in cutting experiment. Control plants were not cut and covered with a net to prevent herbivory. Cut-1 plants had spike I cut when the spike started to appear and then covered with a net. Cut-2 plants were covered with a net and then spike I was cut when the flowers began to bloom. *Phengaris teleius* lays eggs when the spike started to appear, and the larvae feed on the spike when the flowers begin to bloom. Therefore, Cut-2 was assumed as herbivory by *P. teleius*.

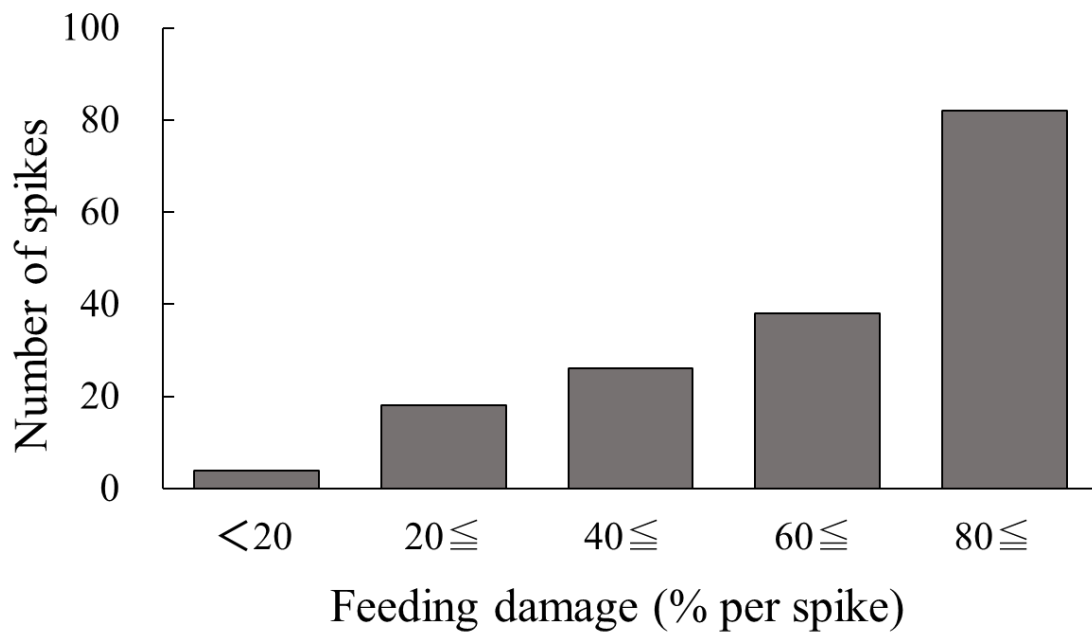


Fig. 1-6 Frequency distribution of feeding damage to spikes by *P. telexus* in natural conditions (n = 168). Feeding damage rate averaged $82.9 \pm 1.7\%$, and approximately half of spikes damaged by herbivory were damaged by 80% or more.

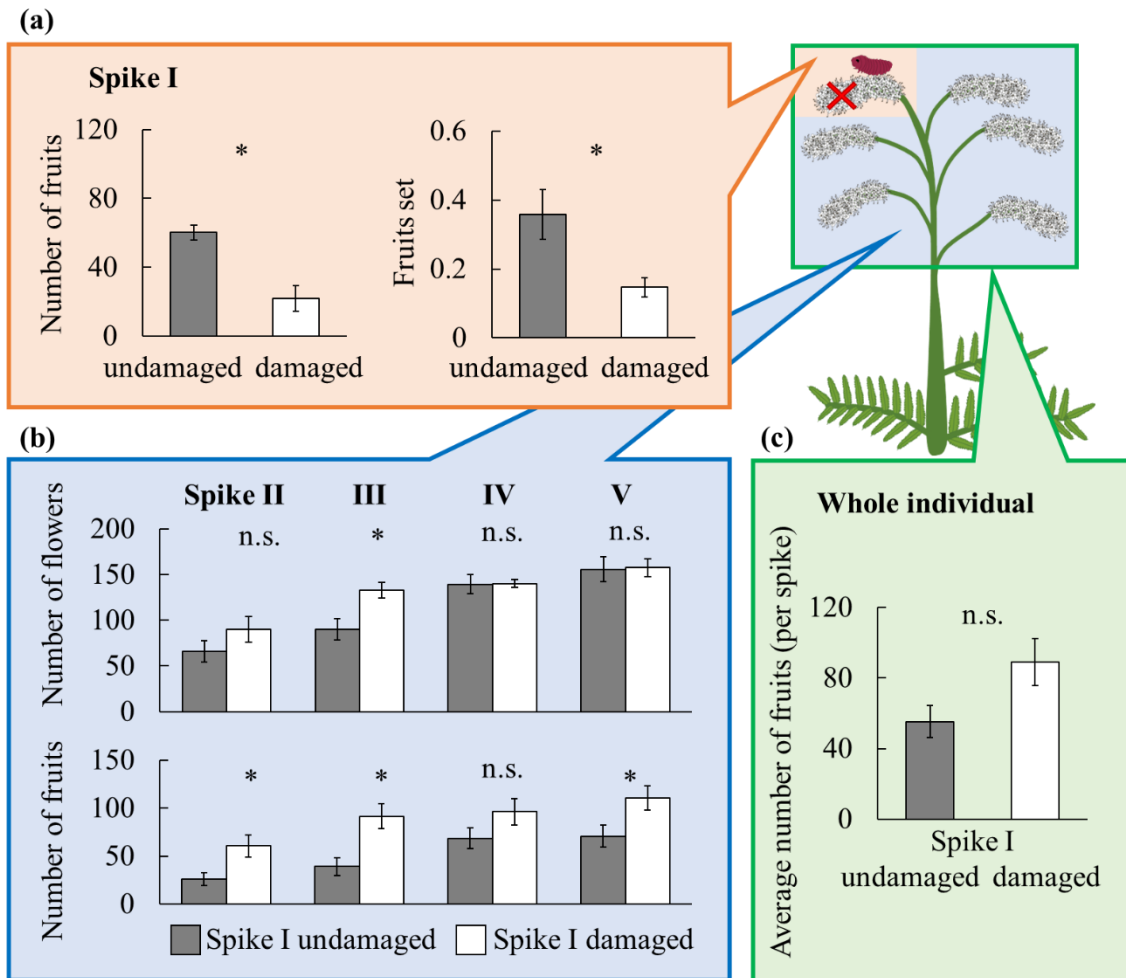


Fig. 1-7 The number of fruits and fruit set between undamaged spike I and damaged spike I (a). The bars show the values of spike without damage (gray) and spike with damage (white). The number of flowers and fruits of spike II-V depending on the presence or absence of damage to spike I (b). The bars show the values of spike without damage (gray) and spike with damage (white) on spike I. Average number of fruits per spike for the whole individual (c). Asterisk indicates the significant differences at $P < 0.05$; n.s. = not significant following t -test.

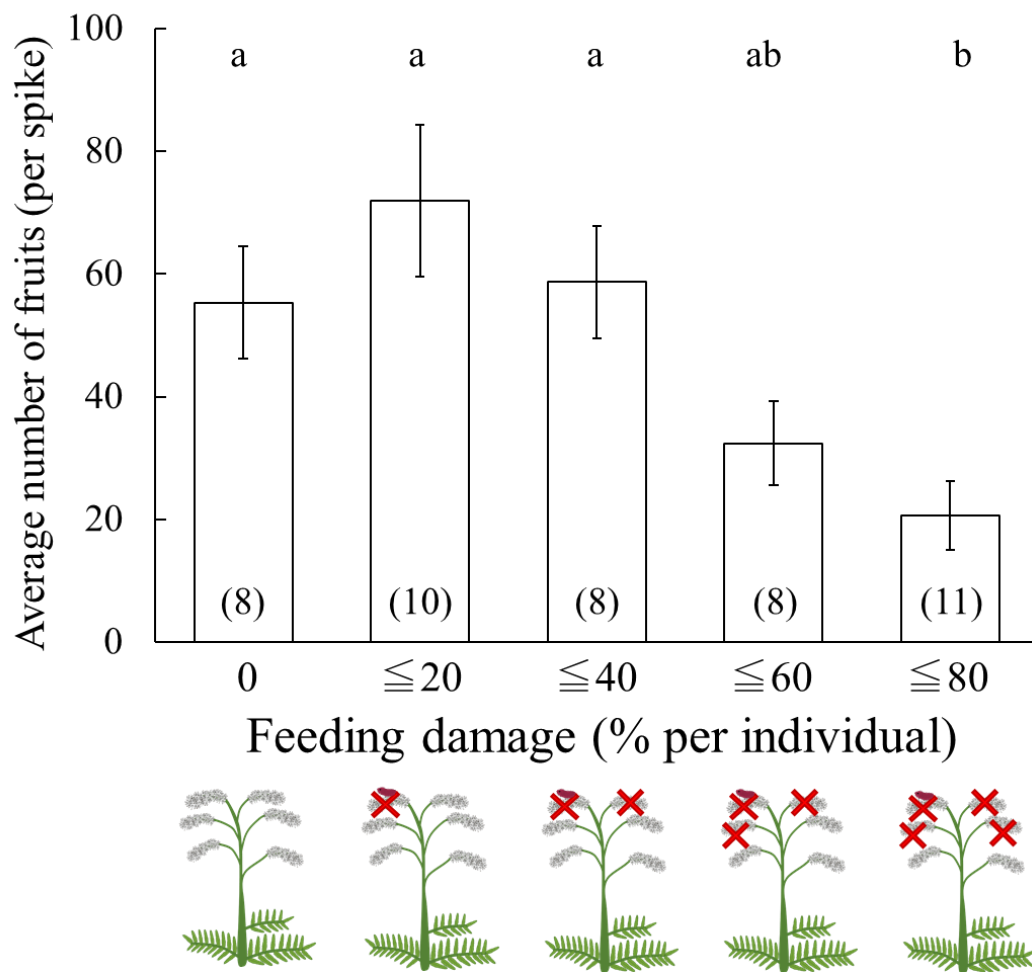


Fig. 1-8 The average number of fruits per spike after feeding damage to the spikes per individual in 2016. The feeding damage rate was divided into 5 categories; 0% (no spikes were damaged), 20% or less, 40% or less, 60% or less, and 80% or less. The number in parentheses is the number of observed individuals. Letters above each bar show the results of the Tukey's test. Same letters show no significance among the values (GLMM; $P < 0.05$).

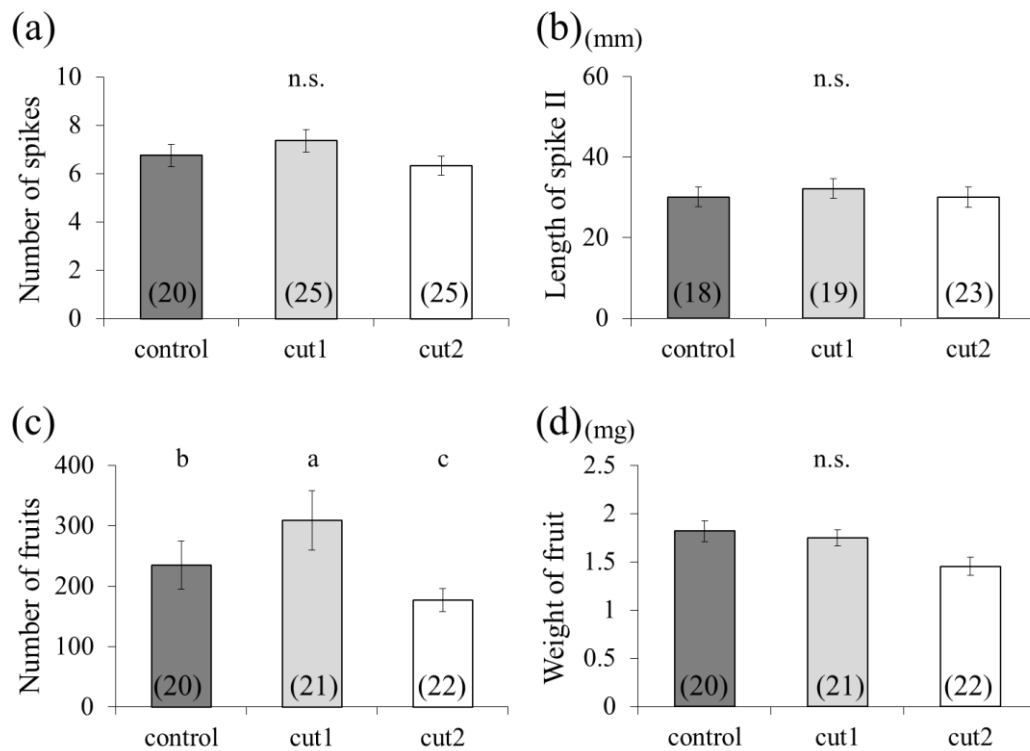


Fig. 1-9 The average number of spikes (a), length of spike II (b), number of fruits (c), and dry weight of fruit (d) in each treatment in 2015. The bars show the values of control (dark gray), Cut-1 (light gray) and Cut-2 (white) plants. There were no significant differences in the number of spikes, length of spikes, or weight of fruits, whereas differences occurred between treatments in the number of fruits (Tukey's test, $P < 0.05$).

Chapter 2. Seasonal and populational comparison of compensatory response of *S. tenuifolia*

2.1 Introduction

Interactions between plants and herbivores have important consequences on the performance of individual plants and plant populations (Marquis 1984). Differences in the size and phenology of plant lead to differences in probability and timing to receive herbivory (Russell & Louda 2005; Wynhoff *et al.* 2014). Because plants can recover from consumption by herbivores in early feeding damage, the effects of herbivory on plant fitness also depend on the timing of the growing period and on the growth stages of the plant (Marshall *et al.* 2005; Oguro & Sakai 2009). As a result, it has been suggested that there are differences in compensatory response to insect herbivory among populations (Huhta *et al.* 2000; Lehndal & Ågren 2015). In addition, spatial and temporal variations in herbivore frequencies related to variation in environmental factors have been observed (Chew & Courtney 1991; Cronin *et al.* 2001). Plants exposed to a high possibility of herbivory, such as populations with large numbers of insect herbivores, may evolutionarily improve in their ability to compensate against herbivory (Lennartsson *et al.* 1997; Juenger & Bergelson 2000). Therefore, the difference in the feeding pressure from herbivores may affect the existence or the effect of compensatory response even in the same plant species.

Sanguisorba tenuifolia grows in various environments such as grasslands and wetlands, roadsides, and mountains (Satake *et al.* 1982). It is reported that the individual size of *S. tenuifolia* and the number of spikes per plant differ among populations (Suzuki 1998). So, it is also important to verify the relationship between compensatory

responses to the morphological variation and frequency and extent of herbivore damage. Similarly, variability in the number of *P. teleius* and flowering phenology of *S. tenuifolia* may cause different results of compensatory response against herbivory by *P. teleius*. To understand compensatory responses of *S. tenuifolia* against herbivory, I should consider the variation among years and among populations.

The purpose of this study was to clarify whether the reproductive compensation of *S. tenuifolia* against herbivory by *P. teleius* exists regardless of the years and of the populations. I tried to verify the constancy of compensation function of *S. tenuifolia* by comparing populations with different case in plant size, flowering phenology, and population size of *P. teleius*. At the same time, I also examine the factors that affect the existence of compensatory response.

2.2 Materials and methods

Study sites

At KHa population, compensatory response against herbivory was detected in 2016 (see Chapter 1). Therefore, similar investigations were conducted from 2017 to 2019 to confirm the existence of compensatory response. In population comparisons, investigations were conducted on nine populations in Hokkaido (Table 1-1, Fig. 1-3). Two populations (IK and TKa) that *P. teleius* do not inhabit were selected as controls to investigate the characteristics of *S. tenuifolia*, which is not affected by the herbivory of *P. teleius*. KHb population is located close to KHa population investigated in Chapter 1, and the individual size of *S. tenuifolia* and the population size of *P. teleius* are similar to KHa population. SP, IK and SO populations are characterized as wetland populations. BB is a population that located on the slope of roadside, and the individual size of *S.*

tenuifolia is relatively large. TKa, TKb and TKc populations are closely located, TKa population occurs at the lakeside, and the other two are roadside populations. HD and KK populations are grasslands in the mountains and are characterized by a small individual size of *S. tenuifolia*. At KHb and KK populations, the investigation was conducted in two years (KHb, in 2018 and 2019; KK, in 2017 and 2018), and at the other populations investigated only one year (Table 1-1).

Annual fluctuations in oviposition preference of P. teleius

In order to investigate the annual fluctuations in oviposition preference of *P. teleius*, I conducted observations of the oviposition behavior of *P. teleius* at KHa population from 2017 to 2019. Observations were conducted from late July to late August (observation days: 2017, 10 times; 2018 and 2019, three times) and *S. tenuifolia* individuals with eggs and/or larvae on the spike were measured the height of flower stem, the length of spike, the position of the spike with eggs and the number of laid eggs. The positions of the spike of individual were numbered as I, II, III, etc., in order from the spike attached to the shoot apex (Fig. 1-4).

Investigation of population size of P. teleius

To investigate the annual variation, I tried to capture *P. teleius* on the selected five days from late July to early September every year when *P. teleius* was active. On each survey date, *P. teleius* were caught in a net for one hour in the morning and 30 minutes in the afternoon. The captured *P. teleius* was identified as male and female, and the wings were marked with an oil-based pen. In order to know the time of occurrence and lifespan of the individual, the color and position of the mark were changed for each

survey date. Similarly, the investigations were conducted in populations other than KHa population, and the population size of *P. telexus* was compared. In addition, 30 *S. tenuifolia* individuals were randomly selected, and 'feeding damage rate of population' was determined based on how many of them were damaged.

Annual and populational variations on characteristics of S. tenuifolia

In each population, I investigated the morphologies and flowering phenology of *S. tenuifolia*. I marked 30 individuals in mid- July. In case, the marked individuals were lost due to deer feeding and/or artificial mowing, I additionally marked 10 to 20 individuals to make up 30 individuals. The marked individuals were monitored three times a month, and the height of flower stems and the number of spikes were measured. For each spike, flowering status was recorded (four categories: blooming, full bloom, withering and fruiting), and the length of spikes was measured at full bloom.

Test of existence of compensatory response

At each population, the spikes were collected after fruiting and the number of fruits and fruit set per spike were measured. Fruit set was calculated as the ratio of the total number of fruits produced to the total number of flowers per spike. At the time of counting the number of fruits, I also checked for feeding damage by *P. telexus*. Feeding damage rate per individual was assessed as the ratio of the number of damaged spikes to the total number of spikes per individual. Feeding damage rate per population was assessed as the damaged individual/total observed individual ratio; the ratio of the number of individuals with at least one damaged spike to the total number of observed individuals.

Statistical analysis

To elucidate the year effects and population effects on morphology, flowering phenology, and fruit production of *S. tenuifolia*, I examined the effects using LMM or GLMM. LMM was used to analyze the height of flower stems, the length of spikes and dry weight of fruits and GLMM was used to analyze the number of spikes, flowering days per spike, flowering start date and the number of fruits (Poisson distribution) and fruit set (binomial distribution). The length of spikes was analyzed by the total length of all spikes and the average length of all spikes. Flowering start date was analyzed by numbering, such as 1 in late July and 2 in early August and so on. In order to exclude the effects of feeding damage caused by *P. teleius*, fruit set, the number of fruits and dry weight of fruits were compared only for the undamaged individuals. The models were analyzed with years or populations as independent variables, each item (height of flower stems, number of spikes, length of spikes, flowering days, number of fruits, fruit set, and dry weight of fruits) as the dependent variable. The marked individuals were included in each model procedures as a random factor. And, Tukey's test was used to compare the differences among years and among populations. To investigate the factors to explain the existence of compensatory response, I performed binomial GLMM with each item as an independent variable, existence of compensatory response as the dependent variable (0-1 data: absence 0, existence 1), and individuals as a random effect. LMM or GLMM were carried out with R 3.5.2 using the 'lmer' or 'glmer' function in the lme4 and Tukey's tests were run with 'ghlt' function in the multcomp packages (R Core Team 2018).

2.3 Results

Annual fluctuations in oviposition preference of P. teleius

As was observed in 2016, *P. teleius* eggs were the most abundant on spike I for three years (41-58%; Fig. 2-1a). In the spikes other than spike I, the ratio was around 10% for each. In 2018, the proportion of eggs laid on the spike I was relatively low, and the eggs laid on the spike VI or later accounted for about a quarter. Of the *S. tenuifolia* individuals that had laid eggs, more than half of them laid only one egg (55-69%; Fig. 2-1b). There were many *S. tenuifolia* individuals with two eggs, and less than 10% of the individuals with four or more eggs.

Population size of P. teleius adults and feeding pressure

As a result of observations, the number of *P. teleius* adults has increased year by year at KHa population (Table 2-1). Compared to 2016, the number of *P. teleius* in 2019 increased by 50 individuals. Feeding damage rate per individual was significantly different between 2018 and 2019 ($P < 0.05$), but others were not. Feeding damage rate per population was relatively low in 2016 and 2018 and was high in 2017 and 2019.

In KHb population, the number of *P. teleius* adults was almost the same as KHa (168 in 2018; 131 in 2019), while that of the other populations was from 1 at BB and TKc to 32 at KK. Feeding damage rate per individual were not significantly different among populations. Excluding populations that are not inhabited by *P. teleius*, feeding damage rate per population ranged from 0.13 to 0.78.

Variations of morphology and flowering phenology of S. tenuifolia

In interannual comparison, there were some differences in the morphology and

flowering phenology of *S. tenuifolia* among years. The number of spikes, the total length of spikes, flowering days per spike, and flowering start date were not significantly different among years ($P < 0.05$; Tables 2-2a, b). The average height of flower stems differed significantly only between 2018 and 2019 ($P = 0.0257$; Table 2-2a), and the average length of spikes was significantly smaller in 2018 ($P < 0.05$; Table 2-2a). Fruit production was greater variation among years. Fruit set, the number of fruits and dry weight of fruits were significantly smaller in 2018 ($P < 0.05$; Table 2-2c). There was no significant difference in all items between 2017 and 2019 ($P < 0.05$; Table 2-2c).

In populational comparison, there were significant differences in all characteristics of the morphology and flowering phenology of *S. tenuifolia* among populations ($P < 0.05$; Table 2-3a, b). The average height of flower stems differed from 56.2 cm at HD to 163.7 cm at TKc, as did the number of spikes differed from 4.7 at HD to 8.4 at BB (Table 2-3a). There was a positive correlation between the height of flower stems and the number of spikes ($P < 0.05$). The total length and average length of spikes were largest at BB (Table 2-3a). Flowering days per spike varied by up to about 10 days among populations, as did flowering start date differed by about a month (Table 2-3b). At the two populations (IS and TKa) where *P. telexus* did not inhabit, the flowering days per spike tended to be relatively short, but no common features were confirmed in the other characteristics. There were significant differences in all characters about fruits production ($P < 0.05$; Table 2-3c). Fruit set differed from 0.06 at SO to 0.45 at KK, and similarly, the number of fruits differed from 3.3 at SP to 42.5 at KK (Table 2-3c). The dry weight of fruits differed from 0.66 mg at KHb to 1.82 mg at KK (Table 2-3c).

Number of fruits of S. tenuifolia and the existence of compensatory response

The number of fruits was compared between the individuals without damaged spike I and the individuals with damaged spike I. Damaged individuals produced more fruit in any survey year at KHa population where interannual comparison was conducted (Fig. 2-2). However, there were annual fluctuations in the amount of fruit increase due to compensation.

The number of fruits was compared between the undamaged and the damaged individuals. At five populations (KHb 2019, BB, HD, KK 2017 and KK 2018), damaged individuals produced more fruit than undamaged individuals (Fig. 2-3). However, at the four populations (KHb 2018, SP, TKb and TKc), the number of fruits remains about the same or reduced. KK population showed an increase in the number of fruits in both years investigated. On the other hand, at KHb population, it did not increase fruits in the first year and increased them in the second year. The populations which investigated for two years had each different result.

The GLMM revealed that existence of compensatory response was positively related to average fruit set of undamaged individuals (Table 2-4). On the other hand, for characteristics other than average fruit set, no significant relationship was detected with existence of compensatory response.

2.4 Discussion

From this study, it became clear that the compensatory response is not a one-year phenomenon at KHa population. In addition, compensatory response was also detected at other populations. Therefore, the compensatory response may be an essential mechanism in *S. tenuifolia* populations. Predispersal seed predation quantitatively

reduce plant reproductive success (Lucas-Barbosa 2016) and directly affect growth rate and density of plant population (Louda 1983; Rose *et al.* 2005). Compensatory response to increase fruit numbers is one of defense strategies for maintaining plant populations. The resources used for fruit production are limited and fruit damage caused by insect herbivores can reduce resource competition among fruits within a plant individual (Stephenson 1981; Obeso 2002). It is considered that the decrease in the number of spikes to which resources are distributed has led to the allocation of more resources to the undamaged spikes and the increase in the number of fruits. However, in any population, *S. tenuifolia* individuals which compensatory response was detected in this study were individuals with a feeding damage rate of about 20% (one or two spikes were damaged). Individuals with a high feeding damage rate, that is, with multiple damaged spikes, produced fewer fruits even with undamaged spikes. This suggests that although the plants have potential ability, the compensatory response does not work in the case of critical and serious damages. To support this situation, it is reported that the ability of plants to compensate against herbivory depends on the amount or severity of herbivory (Tiffin 2000; Fornoni 2011; Blue *et al.* 2015). Furthermore, it is also known that no compensatory response has been shown to occur when damage is severe or prolonged (Huhta *et al.* 2009; West & Louda 2018; Quijano-Medina *et al.* 2019). Since *P. teleius* tend to lay one egg on shoot apex spike of *S. tenuifolia*, so it can be seen that the feeding damage rate is basically suppressed to a level that causes overcompensation. With positive effects of herbivory on plant fitness (such as increase in the number of fruits), it has been hypothesized that overcompensation could be adaptive for plants (Crawley 1987; Hakes & Cronin 2011) and that herbivores may develop a mutualistic relationship with plants (Strauss & Agrawal 1999; Agrawal 2000).

At KHa population, annual fluctuations were observed in the height of flower stem and average length of spike. This difference is thought to be due to an artificial effect, not an environmental factor. This site cuts grass every year around June, when flower stems begin to appear, but the mowing was not done in 2018. Therefore, it is considered that the height of flower stem was higher in 2018. In addition, flowering start date in 2018 was about 10 days earlier than in other years. It is considered that the absence of mowing caused a difference in the morphology and flowering phenology of *S. tenuifolia*. Furthermore, in 2018, the number and quality (i.e., dry weight) of fruits produced declined. Since the phenological mismatch between flowering period and pollinator activity negatively affects seed production (Memmott *et al.* 2007; Kudo & Cooper 2019), it is possible that the shifting of flowering period has caused a difference from the occurrence of insect pollinators.

The differences in the abiotic environment of each population may have affected the differences in the morphology and flowering phenology of *S. tenuifolia*. The individuals in HD and KK populations had low flower stems and a few spikes. These populations are located relatively high altitude with strong wind from the sea. Also in another study (Suzuki 1998), *S. tenuifolia* at higher altitudes had lower flower stems. On the other hand, the morphology and flowering phenology of *S. tenuifolia* population, which *P. teleius* did not inhabit had average values among populations. Insect herbivores damage causes reduced plant size (Züst & Agrawal 2017) and delayed/advanced flowering date of host plants (Valdés & Ehrlén 2017; Sercu *et al.* 2019). However, since the difference of presence/absence or the more/less number of *P. teleius* did not affect the morphology and flowering phenology of *S. tenuifolia*, it is unlikely that inhabiting and herbivory of *P. teleius* affect those of the host plant. The

morphologies and flowering phenology of *S. tenuifolia* were not factors that could explain the existence of compensation. It is known that individual size, i.e., the amount of resource of plant, the phenology overlaps with a pollinator or herbivore, and the timing of herbivory can affect the degree of plant tolerance (Ishizaki *et al.* 2010; Lehdal & Ågren 2015). However, it was found that morphology and flowering phenology did not affect the existence of compensation in *S. tenuifolia*. On the other hand, the capacity of plants to tolerate herbivory also depends on the current environmental conditions (Wise & Abrahamson 2007). Therefore, it is also necessary to compare environmental factors when comparing compensatory response of *S. tenuifolia*.

I revealed that the existence of compensatory response was positively related to 'average fruit set of undamaged individuals'. In the populations with a high 'average fruit set of undamaged individuals', a compensatory response occurred in which the number of fruits of intact spikes increased in individuals damaged. On the other hand, in the population with a low fruit set even in individuals without herbivory, there was a tendency that no increase in the number of fruits in individuals damaged was confirmed. 'Average fruit set of undamaged individuals' is an indicator of fruit production capacity in that environment. Fruit production capacity varies depending on external factors such as weather conditions (Tuell & Isaacs 2010), soil resources (Huhta *et al.* 2009), and pollinators (Garibaldi *et al.* 2013). These external factors can affect the existence of compensatory response.

Table 2-1 Interannual comparison of the number of *P. teiuis* and feeding damage rate of *S. tenuifolia* (mean \pm SE) in KHa population.

	2016	2017	2018	2019
The number of <i>P. teiuis</i>	134	156	177	188
Feeding damage rate per individual	0.43 \pm 0.04	0.45 \pm 0.06	0.32 \pm 0.04	0.55 \pm 0.04
Feeding damage rate per population	0.77	0.9	0.73	0.92

Table 2-2 Interannual comparison of the morphology (a), flowering phenology (b) and fruit production (c) of *S. tenuifolia* (mean \pm SE) in KHa population. The number of observed individuals of *S. tenuifolia* are shown in parentheses. Letters show the results of the Tukey's test and same letters show no significance among the values ($P < 0.05$).

	2017 (29)	2018 (26)	2019 (29)
a. Morphology of <i>S. tenuifolia</i>			
The height of flower stems (cm)	125.5 \pm 4.8 ab	138.4 \pm 3.9 a	122.4 \pm 3.9 b
The number of spikes	6.9 \pm 0.4 a	7.8 \pm 0.6 a	6.7 \pm 0.4 a
The total length of all spikes (mm)	296.6 \pm 21.8 a	249.4 \pm 21.3 a	254.2 \pm 24.4 a
The average length of all spikes (mm)	42.6 \pm 1.7 a	35.2 \pm 2.0 b	40.9 \pm 1.4 a
b. Flowering phenology			
Flowering days per spike	31.4 \pm 1.8 a	29.0 \pm 2.4 a	29.1 \pm 1.7 a
Flowering start date	4.00 \pm 0.16 a	3.04 \pm 0.10 a	4.04 \pm 0.23 a
c. Fruit production (undamaged individuals)			
Fruit set per spike	0.41 \pm 0.09 a	0.12 \pm 0.04 b	0.26 \pm 0.06 a
The number of fruits per spike	39.3 \pm 6.7 a	6.4 \pm 3.1 b	34.5 \pm 10.8 a
The dry weight of fruits (mg)	1.44 \pm 0.17 a	1.18 \pm 0.37 b	1.76 \pm 0.31 a

Table 2-3 Population comparison of the morphology (a), flowering phenology (b) and fruit production (c) of *S. tenuifolia* (mean \pm SE). The number of *S. tenuifolia* individuals observed are shown in parentheses. Since there was no difference between the two years in KHb and KK populations (Tukey, $P < 0.05$), the data for the two years are integrated. Letters show the results of the Tukey's test and same letters show no significance among the values ($P < 0.05$).

	KHb (59)	SP (24)	IK (28)	BB (27)	TKa (22)
a. Morphology of <i>S. tenuifolia</i>					
The height of flower stems (cm)	149.9 \pm 2.7	ab 131.6 \pm 4.6	cd 147.8 \pm 3.9	bc 156.3 \pm 4.0	a 125.0 \pm 7.6
The number of spikes	6.7 \pm 0.2	ab 6.2 \pm 0.4	ac 7.0 \pm 0.3	ab 8.4 \pm 0.4	a 4.9 \pm 0.2
The total length of all spikes (mm)	218.7 \pm 10.8	bc 161.4 \pm 18.8	bc 260.6 \pm 25.2	b 440.7 \pm 26.2	a 166.8 \pm 16.5
The average length of all spikes (mm)	34.6 \pm 1.2	c 35.9 \pm 2.0	bc 39.0 \pm 2.6	bc 53.5 \pm 1.9	a 43.7 \pm 2.3
b. Flowering phenology					
Flowering days per spike	31.0 \pm 1.2	ab 23.6 \pm 1.4	d 23.8 \pm 1.4	d 29.0 \pm 1.0	abc 24.7 \pm 2.1
Flowering start date	1.71 \pm 0.08	c 3.86 \pm 0.24	ab 2.74 \pm 0.14	bc 2.88 \pm 0.17	ab 3.00 \pm 0.24
c. Fruit production (undamaged individuals)					
Fruit set per spike	0.29 \pm 0.04	b 0.07 \pm 0.04	f 0.17 \pm 0.03	de 0.26 \pm 0.05	bd 0.19 \pm 0.03
The number of fruits per spike	22.5 \pm 3.2	b 3.3 \pm 1.6	e 7.3 \pm 1.7	d 23.0 \pm 3.5	b 10.7 \pm 2.1
The dry weight of fruits (mg)	0.66 \pm 0.05	e 1.48 \pm 0.30	abd 1.36 \pm 0.09	bc 0.87 \pm 0.10	ce 0.93 \pm 0.08

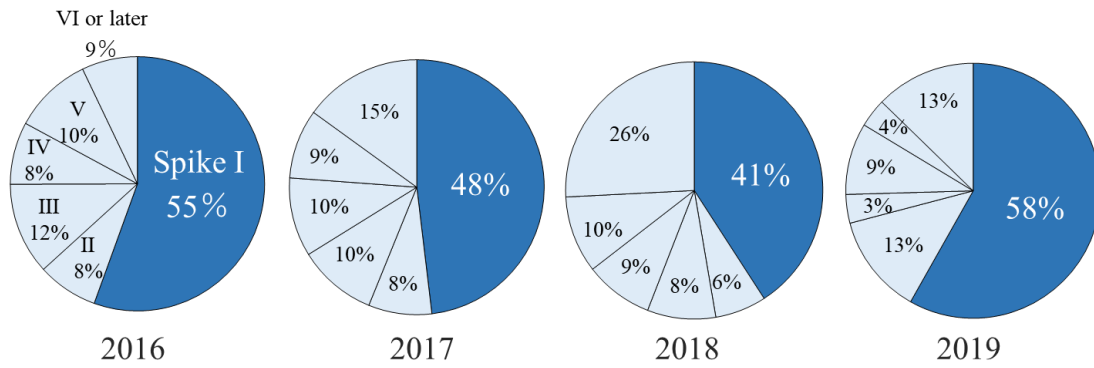
	TKb (22)	TKc (23)	SO (9)	HD (22)	KK (60)
a. Morphology of <i>S. tenuifolia</i>					
The height of flower stems (cm)	154.9 \pm 5.1	ab 163.7 \pm 5.1	a 121.8 \pm 13.5	bd 56.2 \pm 2.4	e 72.1 \pm 1.8
The number of spikes	5.5 \pm 0.4	bc 6.4 \pm 0.3	ac 5.6 \pm 0.6	bc 4.7 \pm 0.3	c 5.7 \pm 0.2
The total length of all spikes (mm)	240.9 \pm 17.8	bc 247.0 \pm 24.2	bc 148.3 \pm 44.3	b 82.9 \pm 13.3	c 249.4 \pm 14.1
The average length of all spikes (mm)	45.4 \pm 2.1	ab 45.9 \pm 1.7	ab 37.2 \pm 3.6	ac 36.2 \pm 3.3	bc 47.8 \pm 1.8
b. Flowering phenology					
Flowering days per spike	33.3 \pm 2.4	a 28.1 \pm 2.2	ad 32.5 \pm 2.5	ad 28.0 \pm 2.0	ad 28.0 \pm 0.8
Flowering start date	3.59 \pm 0.23	ab 2.23 \pm 0.16	bc 3.25 \pm 0.25	ac 2.94 \pm 0.29	ac 4.35 \pm 0.09
c. Fruit production (undamaged individuals)					
Fruit set per spike	0.20 \pm 0.07	cde 0.15 \pm 0.05	ce 0.06 \pm 0.04	cf 0.27 \pm 0.04	b 0.45 \pm 0.03
The number of fruits per spike	12.0 \pm 4.5	c 12.6 \pm 4.1	c 8.2 \pm 6.6	cd 21.5 \pm 4.5	b 42.5 \pm 3.0
The dry weight of fruits (mg)	1.01 \pm 0.23	bce 0.89 \pm 0.14	bce 0.70 \pm 0.15	e 1.05 \pm 0.12	bce 1.82 \pm 0.07

Table 2-4 Factors involved in the existence of compensatory response of *S. tenuifolia*.

P values less than 0.05 are highlighted in bold.

Independent variables	Coefficient	SE	Z-value	<i>P</i> -value
Flower stem height	0.001	0.005	0.222	0.824
Spike number	0.126	0.078	1.609	0.108
Total spikes length	0.002	0.001	1.365	0.172
Average spikes length	0.011	0.015	0.742	0.458
Flowering days per spike	0.034	0.018	1.908	0.056
Flowering start date	-0.128	0.143	-0.890	0.374
Average fruit set of undamaged individuals	2.719	1.209	2.249	0.025
Average fruit weight of undamaged individuals	0.132	0.198	0.666	0.505
Feeding damage rate of population	-1.062	0.713	-1.489	0.136

(a) Position of egg laying



(b) Number of eggs laid per individual

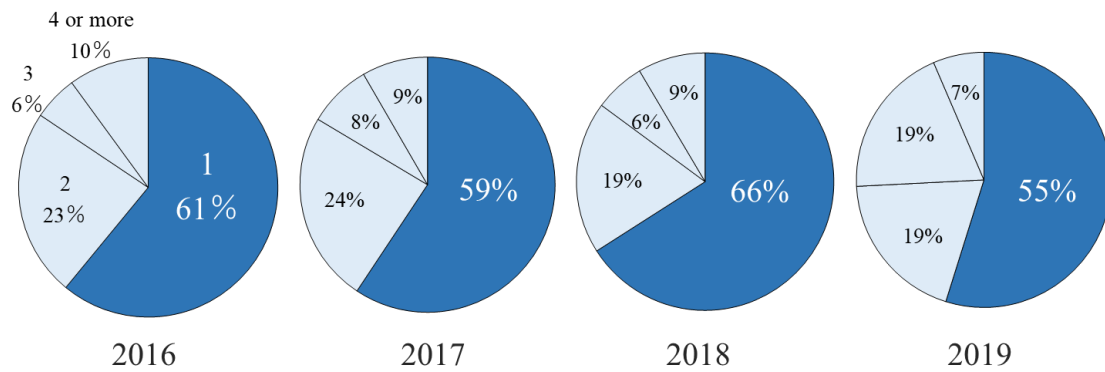


Fig. 2-1 Position of egg laying of *P. teleiuis* (a) and the number of eggs laid per *S. tenuifolia* individual (b). Results are shown for the survey years, 2016, 2017, 2018 and 2019.

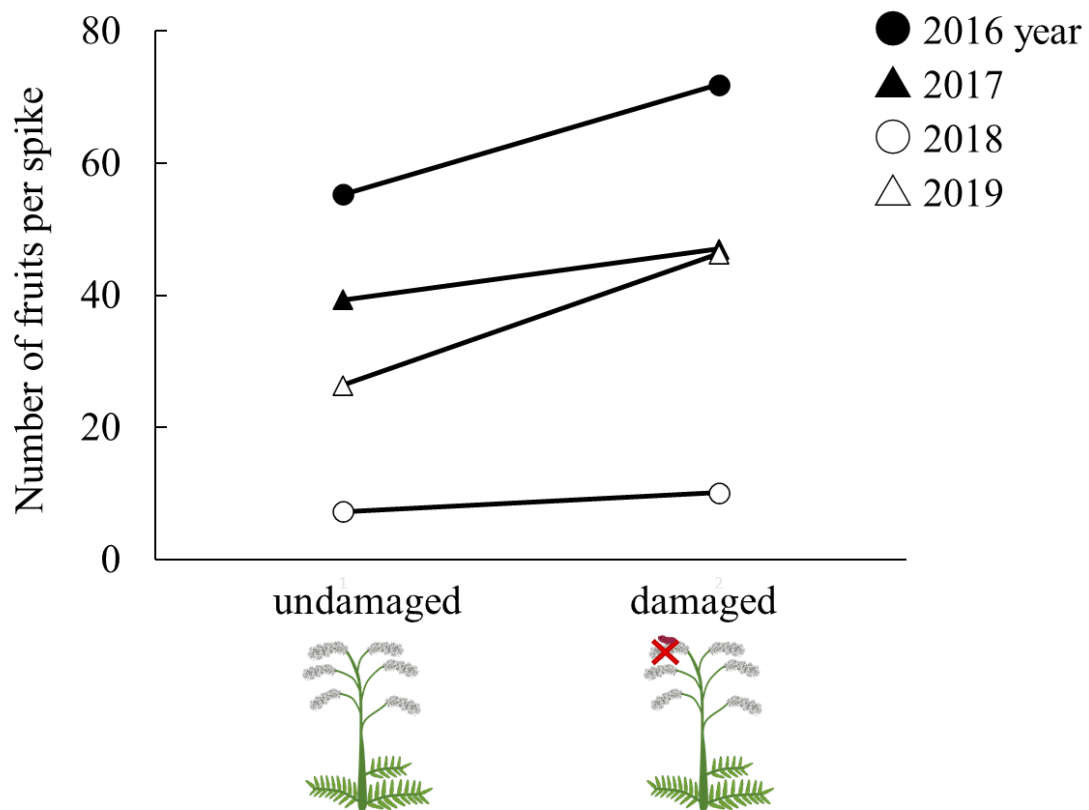


Fig. 2-2 Interannual comparison of the average number of fruits per spike. The left side is individuals without damage, and the right side is individuals with damage on spike I.

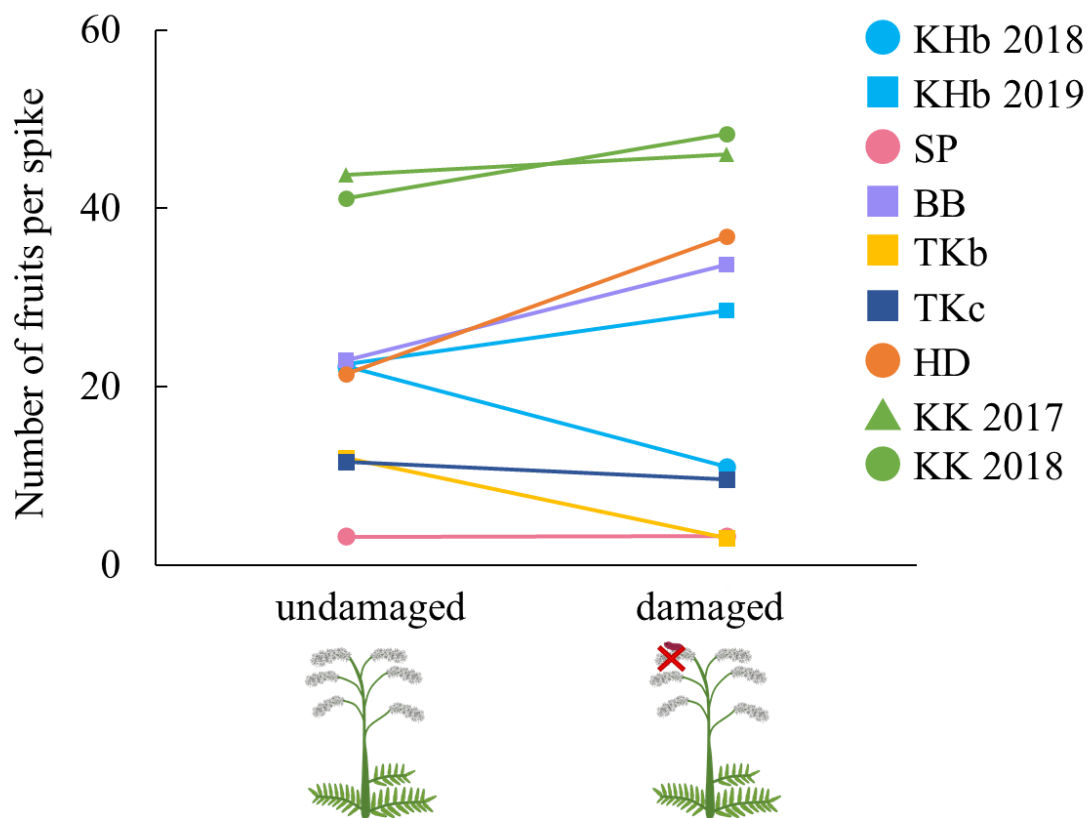


Fig. 2-3 Average number of fruits per spike of the study populations. The left side is individuals without damage, and the right side is individuals with damage on spike I. The color is changed for each population. The shape of marker is changed for each survey year; ▲: 2017, ●: 2018, ■: 2019.

Chapter 3. Effectiveness of *P. teleius* for fruit production of *S. tenuifolia*

3.1 Introduction

Herbivores basically have a negative effect on plant growth and reproduction, while floral visitors as pollinators have a positive effect on seed reproduction even they visit flowers to obtain their food, i.e., pollen and/or nectar (Bronstein 1994). However, there are unique cases that the same insect species shifts its relationship with plants from herbivore to pollinator depending on their life history stages. For example, *Pieris brassicae* feeds on *Brassica rapa* leaves during the larval stage and becomes a pollinator of *B. rapa* when *P. brassicae* becomes an adult (Knauer & Schiestl 2017). In other case, fig wasps (Agaonidae) pollinate *Ficus* plants and lay eggs at the same time, and hatched larvae feed on the seeds for their growth (Kerdelhué *et al.* 2000). Accordingly, it is necessary to evaluate not only the negative effects of herbivory but also the contribution to seed reproduction.

As shown and investigated in Chapter 1 and 2, *P. teleius* has been focused as herbivore of *S. tenuifolia*. However, looking at the life history of both species, the flowering period of *S. tenuifolia* and the appearance period of *P. teleius* adult overlap (Fig. 3-1). By preliminary observations, *P. teleius* adults certainly visit *S. tenuifolia* to obtain floral nectar. Then, when *P. teleius* absorbs nectar, *P. teleius* touches the anthers of *S. tenuifolia* with adhering their bodies. From this, pollens of *S. tenuifolia* may adhere to *P. teleius*, and they may function as a pollinator.

Phengaris teleius is known to visit various flowers such as *Sanguisorba officinalis* and *Viccia cracca* (Sielezniew & Stankiewicz 2013), but its pollinating function has not been evaluated. The purpose of this study was to clarify whether *P.*

teleius adults act as pollinator and contribute seed production on host plants, *S. tenuifolia*. Therefore, it was examined whether pollens were transferred from anthers of *S. tenuifolia* to *P. teleius* and whether pollens were transferred from *P. teleius* to pistils of *S. tenuifolia*. Furthermore, by investigating fruit set of *S. tenuifolia*, the contribution to actual fruit production was verified.

3.2 Materials and methods

Study site

The investigation was conducted at KHa population (Table 1-1, Fig. 1-3). The observations and field experiments were conducted in 2019 and 2020.

Frequency of flower visiting to S. tenuifolia by P. teleius

In 2020 from early August to mid-September which is the flowering period of *S. tenuifolia*, I observed the floral visitor fauna of *S. tenuifolia* to investigate the frequency that *P. teleius* visited the flowers of *S. tenuifolia*. Since *P. teleius* adults fly from sunrise to sunset, I conducted investigations for about one hour each in the morning (Around 9:00 to 10:00), noon (Around 12:00 to 13:00), and evening (Around 15:30 to 16:30). I observed all *S. tenuifolia* individuals in KHa population and recorded the number of *P. teleius* and other insects that stayed in the spikes of *S. tenuifolia* at that time. Insects that species could not be identified were captured by a net and confirmed the species in the laboratory.

In addition, to clarify that *P. teleius* preferentially visit *S. tenuifolia*, I examined the plant species visited by *P. teleius* three times in August for 30 minutes each. Similar investigations were conducted at KHb population, which is geographically close

to KHa population and has a large population size of *P. teleius*, and the plant species visited by *P. teleius* were compared.

Staying time for spikes by P. teleius

In order to investigate the staying time for spikes of *S. tenuifolia* by *P. teleius*, I measured the time from when *P. teleius* started to stay in the spike to when they left. In the morning of mid- August when *P. teleius* was active, I observed the visiting behavior of *P. teleius*. At that time of observations, if *P. teleius* was left from the spike due to the approach of other insects, it was recorded separately as having ‘interference’.

Number of pollens attached from S. tenuifolia to P. teleius

I examined the number of pollens of *S. tenuifolia* attached to *P. teleius* that visited flowers of *S. tenuifolia*. Since pollens may not attach in a short time, *P. teleius* that had stayed in the spikes for 30 seconds or more were captured with a net. And, the scales of the thorax and the abdomen that were likely to touch the anthers of *S. tenuifolia* were collected with cellophane tape. Then, I measured the number of pollens of *S. tenuifolia* using an optical microscope in the laboratory. To discriminate the pollen of *S. tenuifolia*, by collecting and observing the pollen of other 32 plant species at this population in advance, and it was possible to judge that it was pollen of *S. tenuifolia* from the morphology and size of pollen.

Pollen transfer from P. teleius to pistil of S. tenuifolia

I investigated the attaching parts of pollen from *P. teleius* to *S. tenuifolia*. I observed *S. tenuifolia* using fluorescent powder as pseudo-pollen (Fig. 3-2a) to visualize the

attaching of pollens. Fluorescent powder was attached to about 30 individuals of *P. teleius*, and all *S. tenuifolia* individuals in KHa population were observed with black light on the night of the day (Fig. 3-2b). After that, 26 spikes to which the fluorescent powder was attached were randomly collected, and the attaching parts of fluorescent powder were confirmed (Fig. 3-2c).

Difference in frequency of flower visit depending on the existence of herbivory

In the spikes with *P. teleius* larvae, the fruits are consumed due to herbivory, so it is considered that the fruits are not produced even if *P. teleius* visits and pollinates. On the other hand, in the case of spikes without *P. teleius* larvae, it is considered that pollination contribute to fruit production of *S. tenuifolia*. I compared the frequency of flower visit by *P. teleius* depending on the presence or absence of herbivory by larvae of *P. teleius*. 20 *S. tenuifolia* individuals were randomly selected, and the number of *P. teleius* visited each individual was counted four times from mid- August to late August. Each individual of *S. tenuifolia* was directly observed for 10 minutes. After the fruits had matured, the spikes were collected and the existence of herbivory by *P. teleius* was confirmed.

Test of self-pollination using bagging experiments

I conducted pollination experiments to investigate fruit production by self-pollination. 10 spikes in 2016 and five spikes in 2019 were bagged with nylon mesh bags prior to bloom. In 2020, 10 spikes were bagged and forcibly self-pollinated. After the fruit matured, the spikes were collected and fruit set was measured. As a control, it was compared with fruit set of individuals without herbivory by *P. teleius*.

Differences in fruit production of S. tenuifolia depending on the existence of flower visits by P. teleius

I compared the fruit set of *S. tenuifolia* depending on the presence or absence of pollination by *P. teleius*. In order to prevent pollination by *P. teleius*, 30 *S. tenuifolia* individuals were covered with a net in mid- July 2020 (before the occurrence of *P. teleius*). The net is 200 cm in height and 16 mm mesh size which *P. teleius* cannot pass through. As a control, I also prepared 15 individuals without net. Of the individuals that had been netted, 15 individuals remained netted after flowering, and the remaining 15 individuals removed the net after flowering. After the fruit matured, spikes were collected and average fruit set per individual were compared between treatments. The individuals of *S. tenuifolia* that did not bloom during the occurrence of *P. teleius* were excluded from the analysis.

Statistical analysis

I elucidated if *P. teleius* have visit selection for flowers using GLMM. In other words, it was investigated whether there is a difference in the frequency of flower visits by *P. teleius* depending on the existence of herbivory or oviposition. The models were analyzed with the existence of herbivory or oviposition as independent variables, the number of visits by *P. teleius* as the dependent variable (Poisson distribution). Observed individuals (comparison at individual level) and observed spikes (comparison at spike level) were included in each model procedures as random factors. Tukey's test was used to compare the differences in frequency of flower visit. I used GLMM to verify self-compatibility of *S. tenuifolia*. The models were analyzed with fruit set (binomial distribution) and number of fruit (Poisson distribution) as dependent variables and used

Tukey's test to compare the differences among treatments. Observed individuals and survey years were used as random factors. I used GLMM to compare fruit production with and without the visit of *P. teleius*. The model analyzed with fruit set (binomial distribution) as dependent variables and used Tukey's test to compare the differences among treatments. Observation individuals were used as random factors. GLMM were carried out with R 3.5.2 using the 'glmer' function in the lme4 and Tukey's tests were run with 'ghlt' function in the multcomp packages (R Core Team 2018).

3.3 Results

Frequency of flower visiting to S. tenuifolia by P. teleius

Table 3-1 represents the lists of floral visitors of *S. tenuifolia*. In addition to *Phengaris teleius*, ants (Hymenoptera), bees (Hymenoptera), Diptera, Heteroptera and others visited the spikes of *S. tenuifolia*. Of seven observation days, *P. teleius* was a major flower visitor of *S. tenuifolia* on the first two days, i.e., August 8 and 12 (Fig. 3-3). After August 17 with the increment of the flower visitors, more than half of the insects visited *S. tenuifolia* was ants, mostly *Formica japonica* and *Myrmica kotokui*. The number of *P. teleius* visited *S. tenuifolia* decreased on August 26, and almost no visit was observed often September 3. Besides, *P. teleius*, ants and Dipteran insects constantly visited *S. tenuifolia*.

On both populations, more than half of *P. teleius* visited *S. tenuifolia* on all survey days (Fig. 3-4). KHa population had a higher percentage of *P. teleius* visiting *S. tenuifolia* than KHb population. In KHa population, *P. teleius* also visited *Trifolium pratense* and *Eupatorium glehnii*. In KHb population, *P. teleius* also visited *Lespedeza bicolor* and *Lythrum salicaria*.

Staying time for spikes by P. teleius

A total number of *P. teleius* measured the staying time on the spikes was 43 of those, with interference was 13, and with no interference was 30, respectively (Fig. 3-5). The staying time of *P. teleius* with interference was less than 50 seconds with the average of 15 seconds. On the other hand, the staying time of *P. teleius* with no interference varied a few seconds to over 100 seconds with the average of 43 seconds. *Phengaris teleius* with interference left from spikes when other *P. teleius* individuals, *Formica japonica*, *Formica japonica* or hoverflies approached.

Number of pollens attached from S. tenuifolia to P. teleius

Figure 16 represents the data on 22 *P. teleius* individuals that stayed the spikes for more than 30 seconds. Although there was a difference in the number of adherences, pollens of *S. tenuifolia* were attached to *P. teleius* that visited *S. tenuifolia* (Fig. 3-6). Although the number of pollens collected varied from one to over 200 of 22 individuals, 17 individuals were less than 20 pollens attached.

Pollen transfer from P. teleius to pistil of S. tenuifolia

Fluorescent powder was attached to 303 spikes, which was 17.5% of all spikes of *S. tenuifolia* in this population. As shown in Fig. 3-7a of the floral parts, the sepal was the major part that the powders were attached (about 32% overall). For pollination, the attachment to the pistils is essential. Of 26 spikes collected, 22 spikes attached powders on the pistils. The powders were attached to the pistil at a rate of 0 to 40% among spikes (Fig. 3-7b).

Difference in frequency of flower visit depending on the existence of herbivory

At the individual level, there was no significant difference between the number of visits to *S. tenuifolia* with damaged spikes and the number of visits to undamaged *S. tenuifolia* (damaged individuals 6.5 ± 1.1 times, undamaged individuals 6.7 ± 1.9 times; $P > 0.05$; Fig. 3-8a). Similarly, at the spike level, there was no significant difference between the number of visits the damaged spikes and the undamaged spikes (damaged spikes 1.4 ± 0.3 times, undamaged spikes 1.4 ± 0.1 times; $P > 0.05$; Fig. 3-8b). Furthermore, in terms of the presence or absence of oviposition, there was also no significant difference between individuals and between spikes.

Results of fruit production due to pollination experiments

The results of bagging experiments are shown in Fig. 3-9. There were significant differences between bagging and control and between forcible self-pollination and control ($P < 0.05$). Bagging and forcible self-pollination had lower fruit set and produced fewer fruits than controls. There was no significant difference between bagging and forcibly self-pollination (fruit set: $P = 0.505$, number of fruits: $P = 0.734$).

There were no significant differences in fruit set between individuals with visits by *P. teleius* and individuals without visits by *P. teleius* (Fig. 3-10). The fruit set of individuals without visits by *P. teleius* (i.e., individuals covered with a net) was about 13.4%, and the fruit set of individuals with visits by *P. teleius* (i.e., individuals removed a net) was about 11.8%. Controls included individuals with herbivory by scarce large blue and were fruit set of about 9.1%.

3.4 Discussion

More than half of the observed *P. teleius* visited *S. tenuifolia*. This result is common to the two observed populations (KH_a and KH_b) and all survey days, suggesting that *S. tenuifolia* is an important food resource for *P. teleius* adults. On the other hand, as floral visitors of *S. tenuifolia*, the number of individuals of *P. teleius* was less than that of ants. Only days in the early to mid- flowering period of *S. tenuifolia* (August 8 and 12), which were the small number of other floral visitors, *P. teleius* accounted for about half of all floral visitors, but after that, the number of ants increased significantly. *Formica japonica* which accounted the majority of ants observed is known to have a nuptial flight from June to early August, and the eggs laid by a new queen will become worker ants in about a month (Terayama & Kubota 2009). From this, it is considered that the number of worker ants hatched from eggs increased drastically, and the number of ant visits increased during the peak flowering period of *S. tenuifolia*. However, *S. tenuifolia* with bagging and forcibly self-pollination treatments produced little fruit, it was suggested that *S. tenuifolia* has self-incompatibility. Since ants only visit resources near their nest, pollen movements are often limited (Puterbaugh 1998). According to the study of de Vega et al. (2009), it was reported that most of the pollination by ants in *Cytinus hypocistis* (Cytinaceae) is almost self-pollination. Therefore, although ants are the main floral visitors of *S. tenuifolia*, it is considered that pollination by ants have little positive effects on the fruit production of *S. tenuifolia*.

Although *P. teleius* frequently visited *S. tenuifolia*, the number of pollens attached from anthers of *S. tenuifolia* to *P. teleius* and the number of pollens attached from *P. teleius* to pistils of *S. tenuifolia* were both small. From this, it was revealed that *P. teleius* is not an efficient pollinator. Since mid- August conducted the investigations

of number of pollens attached when the number of ant visits are increasing, it is considered that pollens of *S. tenuifolia* were removed by ants before *P. teleius* visits flowers, and the number of pollens attached to *P. teleius* decreased. On the other hand, the small number of pollens attached from *P. teleius* to pistils of *S. tenuifolia* is considered to be related to the position of the pistil. Lepidoptera suck the floral nectar extending their proboscises, so if there are anthers and/or stigmas inside the flower, it is suggested that pollens are unlikely to attach (Bauder *et al.* 2015). The pistil of *S. tenuifolia* is surrounded by four calyxes and four stamens, and is located lower than the stamens. When *P. teleius* stays in the spikes of *S. tenuifolia* for sucking nectar, it is thought that *P. teleius* touches the calyxes and/or stamens but is difficult to touch the pistils. In fact, there was no difference in fruit set of *S. tenuifolia* depending on the existence of flower visits by *P. teleius*. In other words, this suggests that *P. teleius* does not have a positive effect on fruit production of *S. tenuifolia*. It is considered that the low number of pollen transfer between *S. tenuifolia* and *P. teleius* led to the non-contribution to fruit production of *S. tenuifolia* as pollinators of *P. teleius*.

The results of these studies revealed that *P. teleius* and ants are not suitable as pollinators for *S. tenuifolia*. On the other hand, flying insects such as Diptera and Hymenoptera may function as pollinators of *S. tenuifolia*. Diptera and Hymenoptera are also the main pollinators in *Sanguisorba officinalis*, a closely related species of *S. tenuifolia* (Musche *et al.* 2008). However, in investigations of floral visitor of *S. tenuifolia* in 2020, the number of individuals of Diptera and Hymenoptera were smaller than those of ants. The dynamics of pollinator populations affect plant fruit production. From 2016 to 2019, fruit set of *S. tenuifolia* without herbivory averaged 31.8% (see Chapters 1 and 2), while fruit set of *S. tenuifolia* without herbivory in 2020 was 11.8%

(see Fig. 3-10). The low fruit set in 2020 may have been influenced by the small number of individuals of Diptera and Hymenoptera that function as pollinators. In the future, verification of the role of Diptera and Hymenoptera as pollinators in *S. tenuifolia* will be necessary for understanding fruit production of *S. tenuifolia*.

In addition, the relationship between visiting preference of floral visitors and compensatory response of *S. tenuifolia* should be considered. In the case of *P. teleius*, there was no difference in the frequency of visits to *S. tenuifolia* depending on the existence of herbivory. However, from the results of Chapters 1 and 2, it was revealed that individuals of *S. tenuifolia* with damaged spikes, compensatory response occurs in which the number of fruits produced by the remaining undamaged spikes increases. In some plants, compensatory response has been observed in which the number of visiting insects increases in individuals with flowers damaged and more frequent visits to the remaining intact flowers (Krupnick *et al.* 1999; Cozzolino *et al.* 2015). As well in *S. tenuifolia*, the change in the number of visiting insects depending on the existence of herbivory may have affected the fruit production (and thus compensatory response) of *S. tenuifolia*.

Table 3-1 Flower visiting insects of *S. tenuifolia*. On each survey day, the investigations were conducted three times: M, morning; N, noon; E, evening.

Weather	Aug. 8			Aug. 12			Aug. 17			Aug. 22			Aug. 26			Sep. 3			Sep. 19			Total		
	M	N	E	M	N	E	M	N	E	M	N	E	M	N	E	M	N	E	M	N	E			
Wind direction & wind power	cloudy SW2	sunny SW2	sunny SW2	clear SSE3	sunny SE2	sunny SSE1	clear SE1	S2	SE3	sunny N2	SE2	S2	sunny S2	S3	S2	cloudy SE3	SE4	SE4	sunny NW2	sunny W2	cloudy ESE3			
Temperature	20	22	25	27.5	31	35	26	29.5	24.5	20	25	26	26	29	30	25	26.5	23	22	22	21			
<i>Phengaris teleius</i>	18	33	8	30	56	5	59	81	5	42	68	38	19	32	16	3	3	0	0	0	0	0	0	0
Ants	6	6	15	14	21	29	91	134	97	143	163	198	261	349	332	301	302	63	100	275	181	3081		
<i>Myrmica kotokui</i>	4	5	9	2	4	6	3	3	2	1			1	3	4				11	60	36	151		
<i>Formica japonica</i>	2	1	6	12	16	23	91	131	94	142	163	197	261	348	329	297	302	63	89	215	145	2927		
<i>Lasius japonicus</i>				1			1			1												3		
Bees	1	0	0	2	3	3	5	2	2	3	6	6	8	7	0	16	4	0	6	4	2	80		
<i>Vespa sinillima xanthoptera</i>							1												2			3		
<i>Bombus deuteronymus deuteronymus</i>																						0		
<i>Apis mellifera</i>										1			2			5			3	1		12		
Sphecidae																1						0		
Halictidae 1										1												1		
Halictidae 2										1												1		
Halictidae 3										1			4			8			1	2	1	28		
Chalcididae																						0		
Hymenoptera 1																						0		
Hymenoptera 2																						0		
Hymenoptera 3																						1		
Hymenoptera 4																						14		
Hymenoptera 5																						15		
Hymenoptera 6																						1		
Hymenoptera 7																						1		
Hymenoptera 8																						1		

Table 3-1 continued

Diptera	12	5	4	13	6	9	27	23	8	37	33	11	49	48	23	45	22	6	41	30	47	499	
Calliphoridae	1						3	2		11	12	1	7	3	1	9		1	2	1	1	1	55
<i>Stomorphina obsoleta</i>									1	1	1	3	5	16	10	1	2		2	1	6	10	
<i>Sarcophaga similis</i>							1		1	1	1	3	2	1	1	1	2		7	5	17	79	
Anthomyiidae													2	1		1					1	5	
Tachinidae 1				3	1	1	3	1	2	7	7	5	7	8	6	6	6	1	1	2	5	72	
Tachinidae 2								1		2					2	1					5	5	
Tachinidae 3											1					3	5	1	1	1	1	13	
Tachinidae 4										5						1	2		3	3	4	18	
<i>Tachina nupta</i>										1									1			1	
<i>Tachina</i> sp.																			1			1	
<i>Dexosoma caninum</i>																				1	1	2	
<i>Phasia sinensis</i>																2			2	2	2	6	
<i>Episyrphus balteatus</i>																			2			2	
<i>Sphaerophoria philanthus</i>	4	1						1		3	1		7			1	1			1		20	
<i>Platycheirus clypeatus</i>																	1		1			2	
<i>Epistrophe (Epistrophe) nitidicollis</i>																			1	2	1	4	
<i>Chrysotoxum shirakii</i>							4						3			1	1		2			9	
<i>Syrirta pipiens</i>	2	1											1	2	1	1			5	8	2	23	
<i>Phytomia zonata</i>													2	1		2						5	
<i>Eristalis cerealis</i>						1	2	1		1			1									6	
<i>Ferdinandea cuprea</i>										2												2	
<i>Tabanus sapporoensis</i>					1				2							1						4	
Cheilosini gen. sp.	7	1	2	6	1	3	10	10	2		6	1	9	16	2	4	2		11	5	5	103	
Syrphinae																						1	
Syrphidae 1										3			2			2						7	
Syrphidae 2	1						2	2					1			1						7	
Brachycera 1							2				1											3	
Brachycera 2								1														1	
Brachycera 3													1									1	
Brachycera 4				1									1									2	
Brachycera 5																						0	
Brachycera 6			1	2	3	4		1	1	1	2	1		1	1					1		19	
Brachycera 7				1													1					2	

Table 3-1 continued

Brachycera 8																					2	
Brachycera 9																					1	
Brachycera 10																					5	
Brachycera 11																					1	
Heteroptera																						92
<i>Dolycoris baccarum</i>																						1
<i>Graphosoma rubrolineatum</i>																						25
<i>Megalotomus costalis</i>																						12
Coreidae																						29
Miridae 1																						13
Miridae 2																						8
Others																						5
<i>Popillia japonica</i>																						146
<i>Cetonia (Eucetonia) pilifera</i>																						9
<i>Coccinella septempunctata</i>																						4
<i>Propylea japonica</i>																						8
<i>Agrypnus fuliginosus</i>																						4
<i>Monochamus subfasciatus</i>																						1
<i>Symplectra paedisca</i>																						1
<i>Symplectrum darwinianum</i>																						0
<i>Symplectrum frequens</i>																						1
<i>Symplectrum infuscatum</i>																						5
<i>Oecanthus longicauda</i>																						2
<i>Euclidia dentata</i>																						7
Chrysomelidae																						1
Staphylinidae																						3
Elateridae																						0
Chrysopidae																						35
Salticidae																						23
<i>Ebrechella</i> sp.																						29
<i>Thomisidae</i> sp.																						11
																						2
	43	53	38	65	96	68	192	252	126	232	282	263	350	443	378	382	336	72	154	344	245	4414

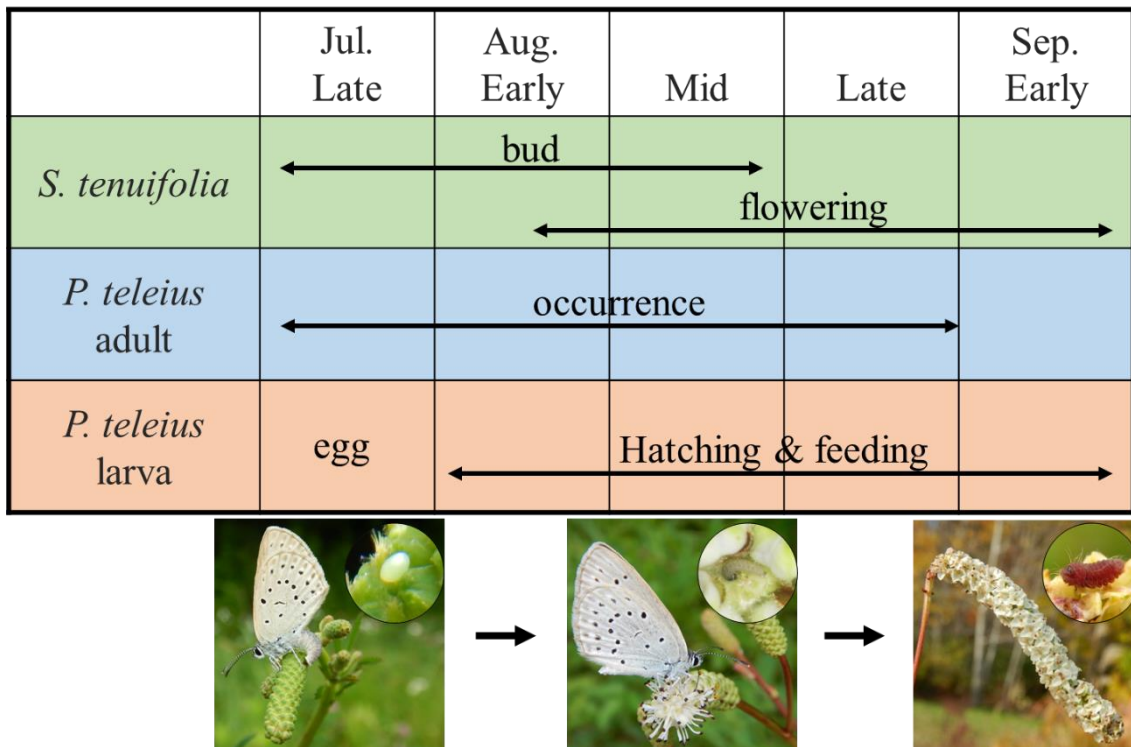


Fig. 3-1 Phenology of *S. tenuifolia* and *P. teleius*. The upper row shows the flowering phenology of *S. tenuifolia*. The phenology of *P. teleius* adult and larva are shown separately. The middle row shows the time of occurrence of *P. teleius* adult and the lower row shows it of *P. teleius* larvae. The flowering period of *S. tenuifolia* and the time of occurrence of *P. teleius* adult overlap.

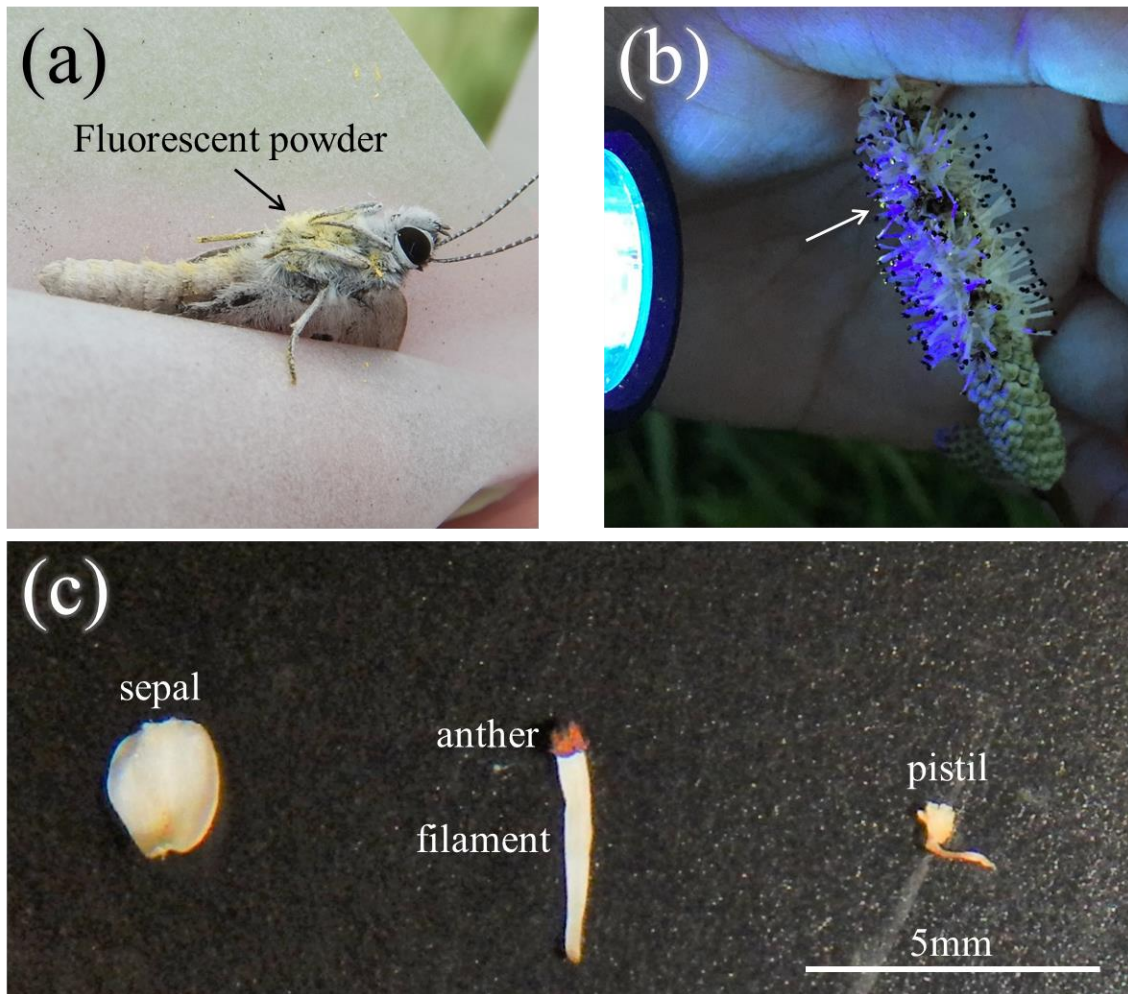


Fig. 3-2 Photographs of *P. teleius* with fluorescent powder (a), fluorescent powder illuminated with black light (b), and each part of *S. tenuifolia* florets (c). Fluorescent powder is indicated by an arrow.

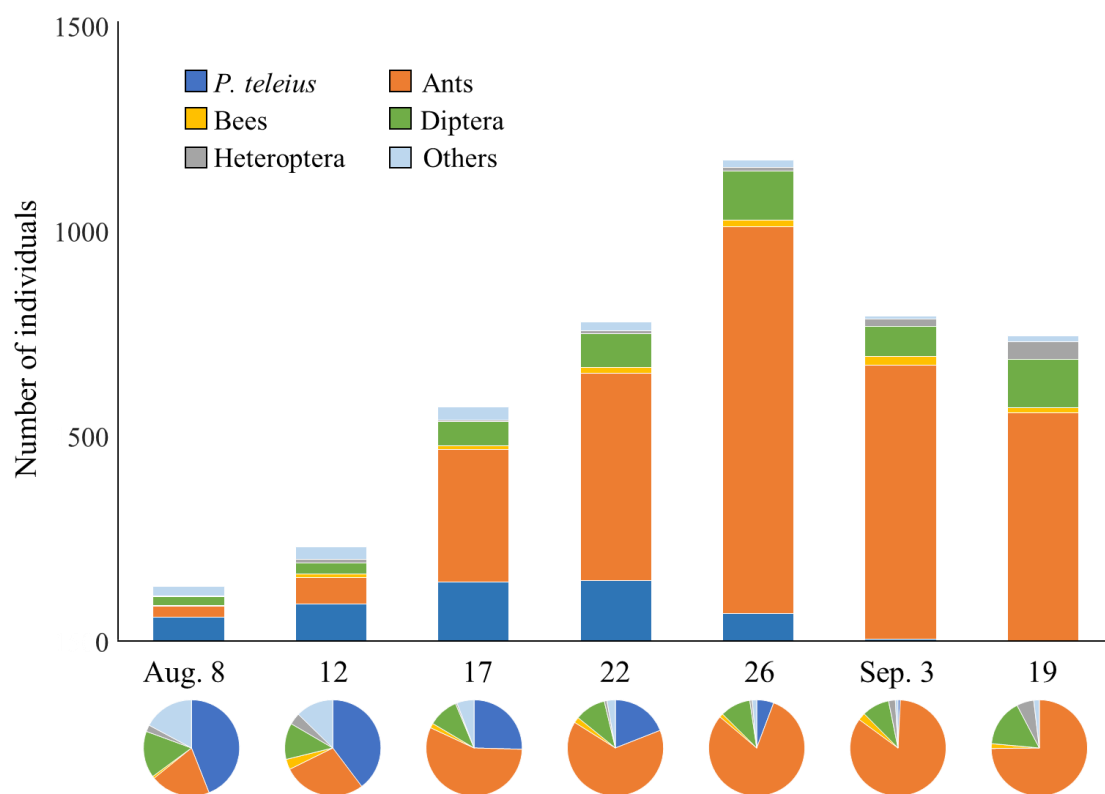


Fig. 3-3 Flower visiting insect fauna of *S. tenuifolia* for each survey day in 2020. The bar graph shows the number of insect individuals, and the pie chart shows the percentage. Three times of the data (morning, noon and evening) of each survey date have integrated. The colors of the graph are distinguished by insect species or taxon.

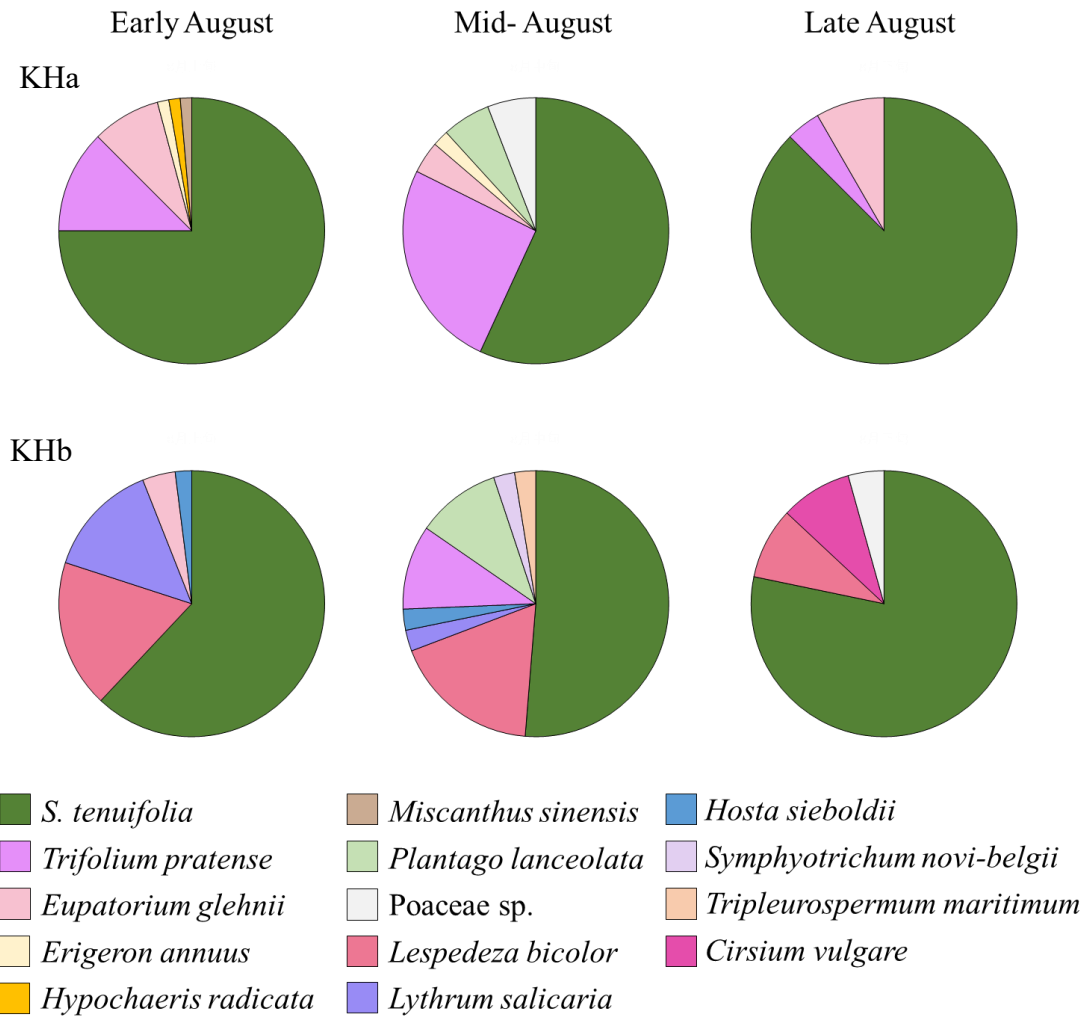


Fig. 3-4 Percentage of plant species visited by *P. teleius*. The upper row shows the results of KHa population, the lower row shows the results of KHb population, and from the left to right, the results are for early August, mid-August, and late August. The colors are distinguished by plant species. Only *P. teleius* individuals that touch the place with flowers are counted, and individuals that stay on the leaves or stems are excluded.

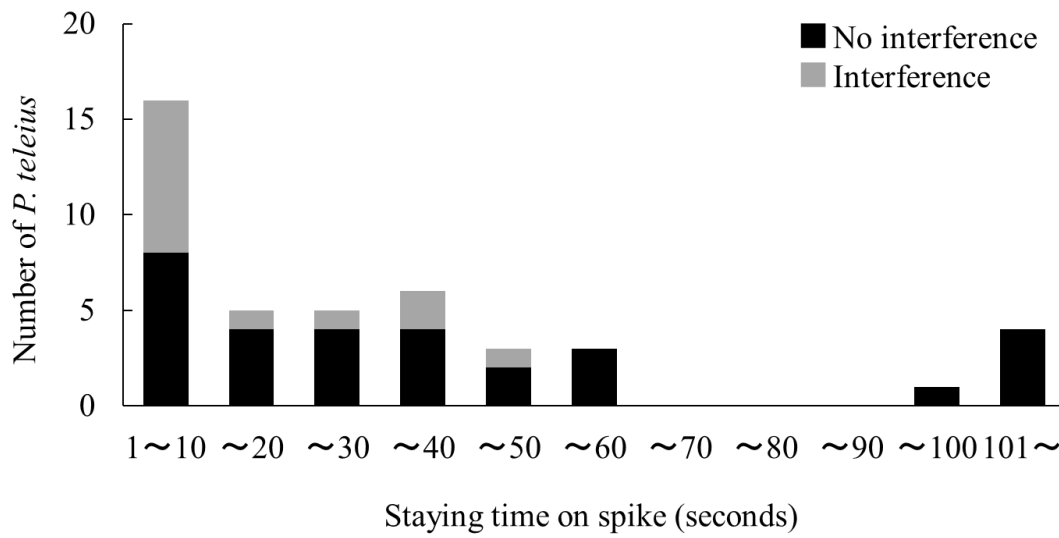


Fig. 3-5 Time that *P. teiei* stayed on *S. tenuifolia* spikes. The horizontal axis shows the staying times, and the vertical axis shows the number of *P. teiei* that stayed for that time. Black bar indicates the number of individuals that were not disturbed (such as the approach of other insects), and gray bar indicates the number of individuals that were disturbed.

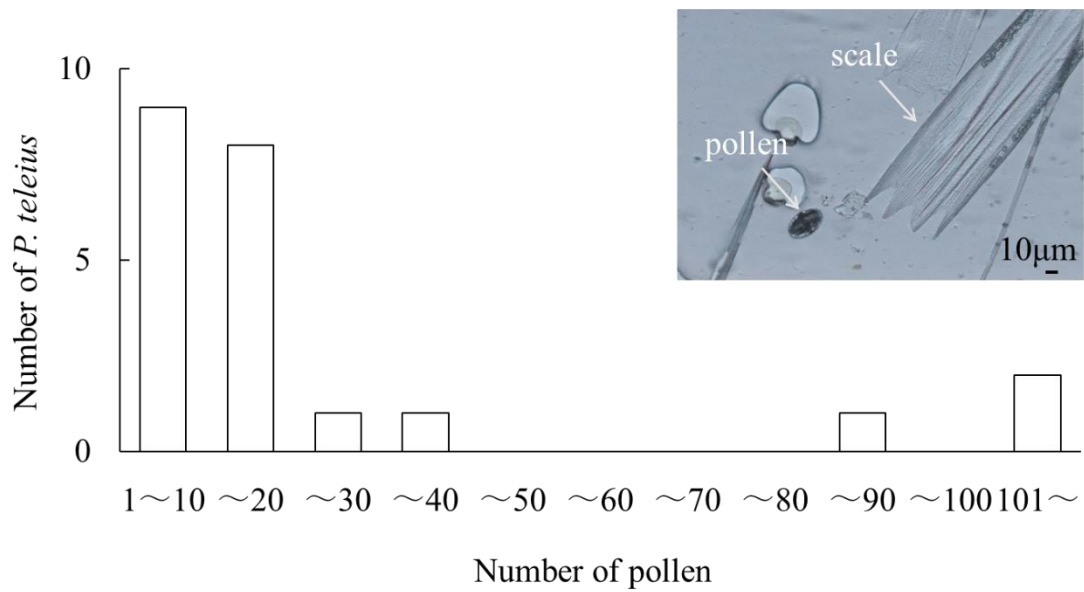


Fig. 3-6 The number of pollen of *S. tenuifolia* attached to *P. teleius*. The horizontal axis shows the number of pollen, and the vertical axis shows the number of *P. teleius* that attached the pollen. The upper right is a photograph of the scales of *P. teleius* and the pollen of *S. tenuifolia*. Photograph was taken using an optical microscope of scales collected with cellophane tape.

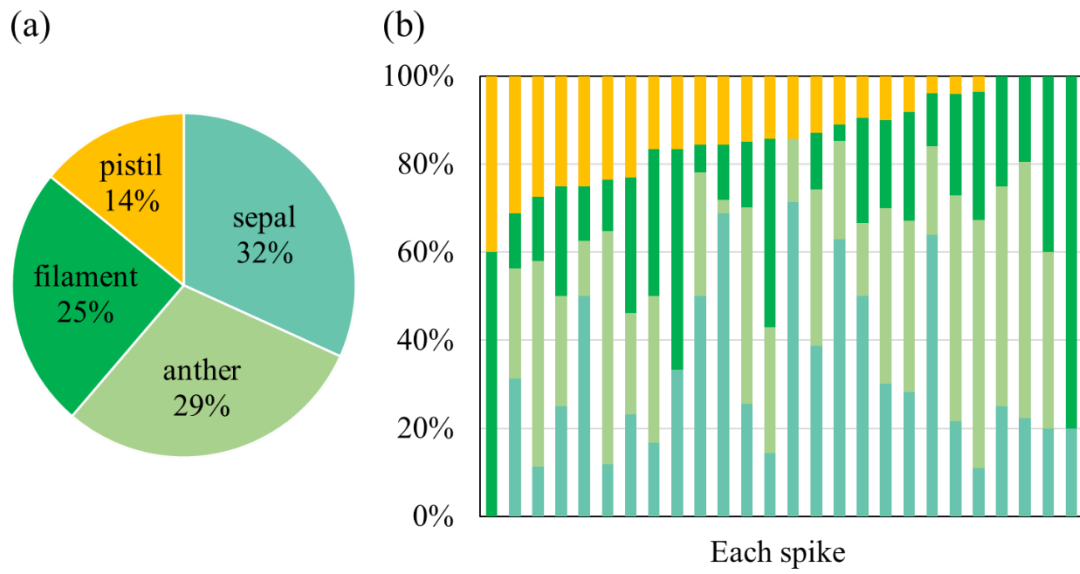


Fig. 3-7 Total proportion of each part of *S. tenuifolia* florets to which fluorescent powder was attached (a) and those proportion of each spike (b). The colors are distinguished by the part of the florets. The percentage of the attaching parts of 26 spikes collected is shown in each bar graph.

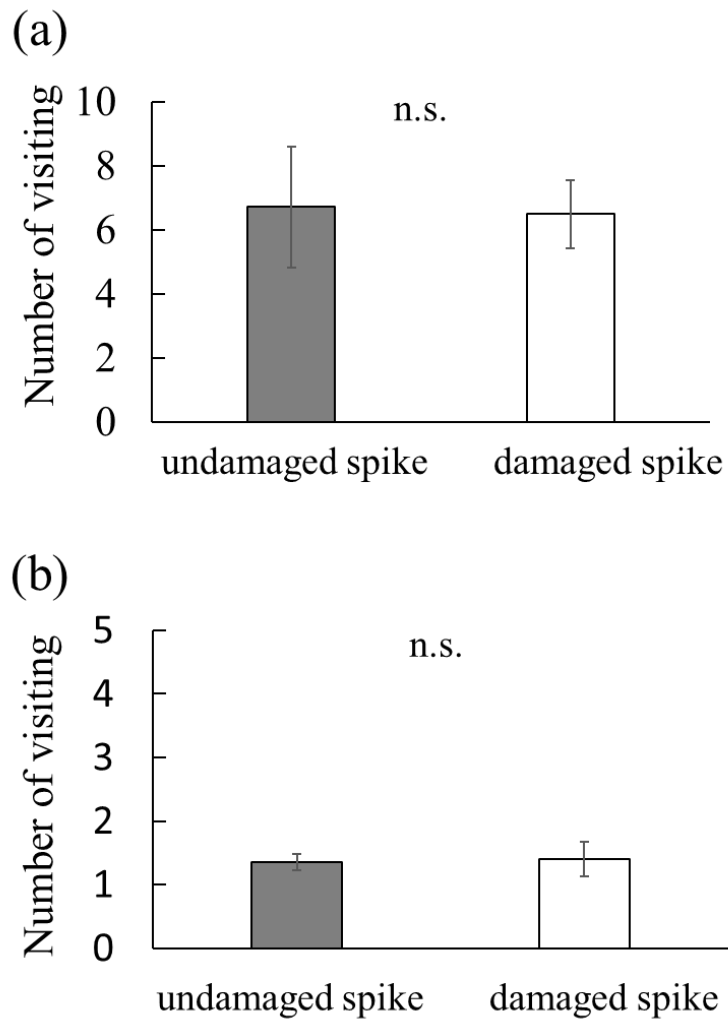


Fig. 3-8 The number of flower visits of *P. telexus* between individuals with herbivory and individuals without herbivory (a), and between spikes with herbivory and spikes without herbivory (b). White bars indicate individuals or spikes that have been damaged, and gray bars indicate individuals or spikes that have not been damaged. No significant difference was detected between with and without herbivory in Tukey's test (GLMM; $P < 0.05$).

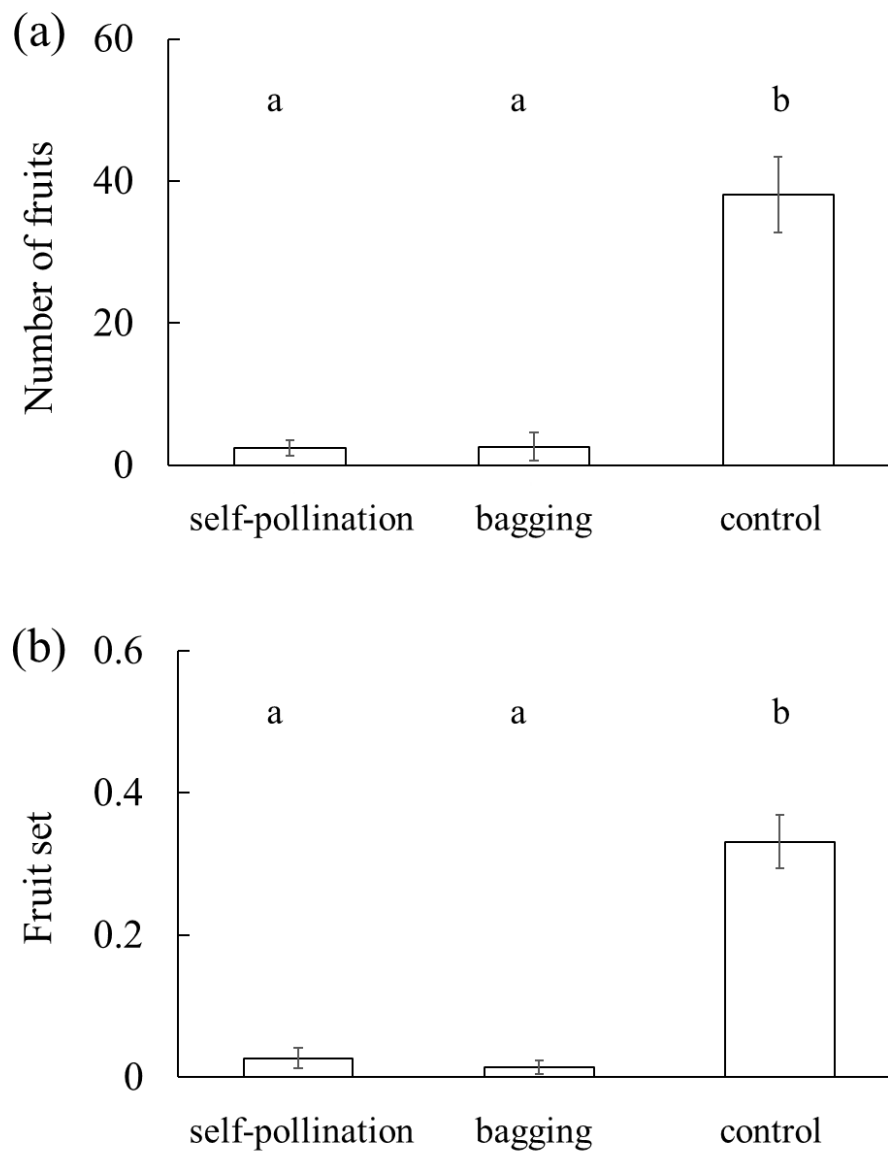


Fig. 3-9 The number of fruits produced (a) and fruit set (b) by treatments of bagging and forced self-pollination. From left to right, the bars show bagging and forced self-pollination, only bagging, and control. Letters above each bar show the results of the Tukey's test. Same letters show no significance among the values (GLMM; $P < 0.05$).

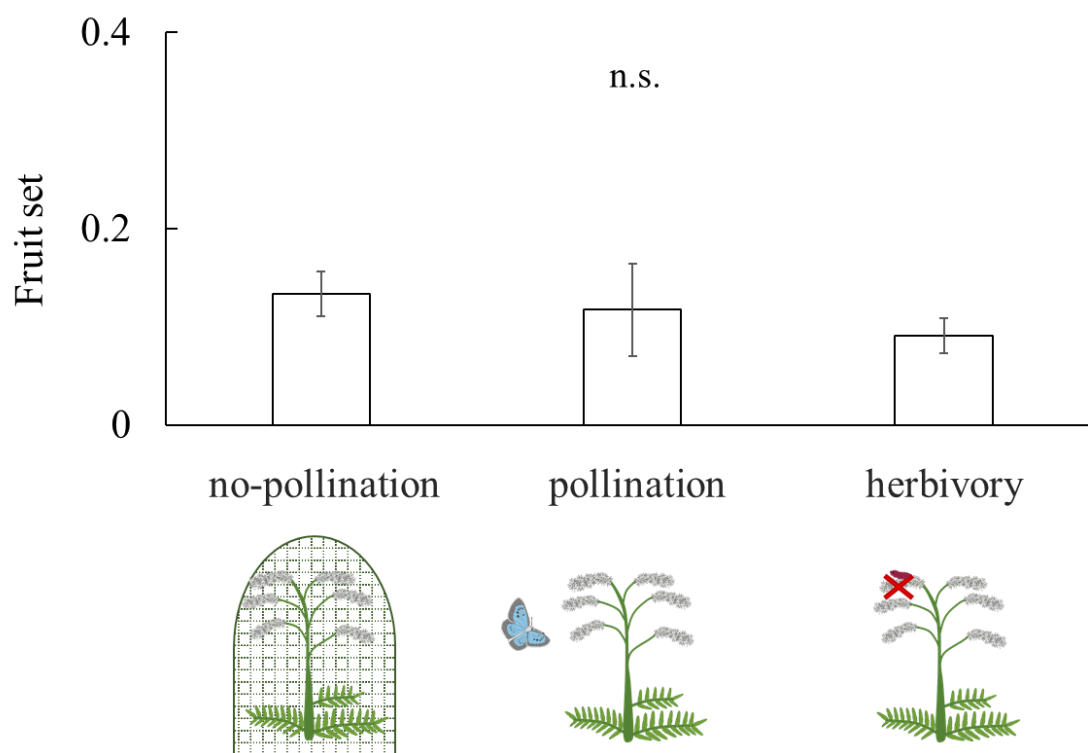


Fig. 3-10 Fruit set depending on the presence or absence of flower visit of *P. teleius*.

From left to right, the bars show individuals without visits by *P. teleius* (i.e., individuals covered with a net), individuals with visits by *P. teleius* (i.e., individuals removed a net), and control individuals (i.e., individuals without a net). Control individuals also include individuals that are damaged by *P. teleius*. No significant difference was detected between with and without flower visits in Tukey's test (GLMM; $P < 0.05$).

General discussion

In Chapter 1, I clarified that *Sanguisorba tenuifolia* has a compensatory response to supplement fruit production against herbivory of *Phengaris teleius*. The oviposition of *P. teleius* was basically limited and did not result in excessive feeding pressure on *S. tenuifolia*. As a result, the remaining spikes that were not damaged by herbivory increased the number of fruits.

In Chapter 2, I confirmed the consistency of defense mechanism of *S. tenuifolia* against herbivory by conducting verification in multiple years at the same population and various populations. Compensatory response has been observed for multiple years at KHa population, suggesting that *S. tenuifolia* has the ability of compensation to the herbivory by *P. teleius* as a stable mechanism within the population. Compensatory response also existed in other populations by comparing fruit production among different populations, such as individual size, flowering phenology of *S. tenuifolia*, and number of *P. teleius*, due to differences in habitat.

In Chapter 3, I clarified that floral herbivore *P. teleius* does not contribute to pollinating in host plants. *Phengaris teleius* adult frequently visited *S. tenuifolia* flowers. In actual fruit production of *S. tenuifolia*, since fruit set of *S. tenuifolia* did not differ regardless the existence of visiting by *P. teleius*, suggesting that *P. teleius* does not contribute to *S. tenuifolia* as a pollinator. That is, *P. teleius* has only a role of parasitic interaction as a floral herbivore of *S. tenuifolia*.

Plants, herbivores and pollinators respond each other differently under spatial and temporal variations in environmental factors that may affect their synchrony and intensity of interactions (König *et al.* 2015). Weather conditions, such as temperature, wind, and precipitation, may make a direct difference in intensity of interactions among

and within years (Peñuelas *et al.* 2002). Differences in the number of fruits or seeds produced among years are related to abiotic factors such as weather conditions (García *et al.* 2002; Ågren *et al.* 2008) and biological factors such as pollinator insects (Memmott *et al.* 2007; Kudo & Cooper 2019). No compensatory response was detected in some populations, which is not due to the absence of compensatory mechanism, but there may be not in a state where they could be compensated.

Although most butterflies feed on nectar, their role as effective pollinators remains questionable (Bauder *et al.* 2015). Since *P. teleius* did not contribute to pollination of *S. tenuifolia*, they had no positive effect on fruit production of the larval host plant. In some interspecific relationships, such as fig-fig wasps (Kerdelhué *et al.* 2000) and *Phyllanthae-Epicephala* (Kawakita 2010), the seed herbivores also play the role of pollinator. These interspecific relationships are obligate pollination mutualisms, which is established because both species are specialists. On the other hand, *S. tenuifolia* is a generalist which is pollinated by various insect species such as Diptera and Hymenoptera, suggesting that *P. teleius* is not efficient pollinator of *S. tenuifolia*. Therefore, the effect of *P. teleius* on the fruit production of *S. tenuifolia* is that there is no positive effect by pollination, and it became clear that the relationship between the two species is maintained by compensatory response against herbivory.

Floral herbivores have a direct negative effect on seed reproduction (Strauss 1997). Previous studies have also confirmed an increase in the number of seeds and/or seed set in other flowers within a damaged individual (Liao *et al.* 2013; West & Louda 2018). However, these are the experiments conducted at only one or two populations, and a comparative study of compensatory response focusing on the individual size, flowering phenology, fruit production capacity, and abundance of floral herbivores of

the target plant was not efficiently done. Therefore, this study was able to verify the existence of the compensatory response against feeding damage of floral herbivores as well as the constancy of the compensatory response among the populations. The elucidation of defense mechanism against herbivory and reproductive strategies of *S. tenuifolia* has provided important insights not only for maintaining population of *S. tenuifolia*, but also for maintaining population of *P. teleius* endangered around the world.

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