

Relationships among Flower Color Polymorphism, Feeding by Visiting Insects, and Seed Production in *Hepatica nobilis* var. *japonica*

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Abstract To clarify the relationship between the flower color polymorphism maintained in a population and flower visitors as pollinators or herbivores, we observed visiting insect fauna, focusing on visual color discrimination ability and feeding damage to bluish-purple, reddish-purple, and white *Hepatica nobilis* var. *japonica* flowers. Although clear differences in the color recognition abilities of Hymenoptera and Diptera by color model estimate were confirmed for white and pigmented flowers, no difference was found in the number of visits by these orders. By contrast, although there is some annual variation in visitation rates, Coleoptera visited more white flowers than pigmented flowers. In addition, bluish-purple flowers were the least often damaged by herbivores. Although the plants with flowers with damaged tepals, stamens, or gynoecia produced significantly fewer seeds, the total numbers of seeds did not differ among flower colors, presumably because herbivores also act as pollinators, and may promote autogamy. These results indicate that herbivores had antagonistic functions in terms of feeding damage and pollination, and their influence differed by flower color type. Taken together, these data suggest that the mechanism by which flower color variation is maintained may include both opposing and fluctuating selection.

Key words: flower color polymorphism, *Hepatica*, herbivore, pollinator.

Introduction

To understand the maintenance mechanism of flower color polymorphism in a population, the consideration of how flower color morphs relate to visiting insects is important. Based on previous studies, two hypotheses have been proposed. One hypothesis involves the relationship with pollinators (e.g., Stanton, 1987; Jones and Reithel, 2001). Although pollinators often have

strong preferences for certain flower colors (Spaethe *et al.*, 2001; Rausher, 2008), Gigord *et al.* (2001) showed that in rewardless plant *Dactylorhiza sambucina*, the color of the minority became reproductively advantageous as visiting insects learn that majority colors are rewardless. In this case, polymorphism would be maintained by negative-frequency dependent selection. The other hypothesis involves defense against herbivores. Several studies have examined the rela-

tionship between flower color polymorphism in populations and herbivores (e.g., Irwin *et al.*, 2003; Carlson and Holsinger, 2013; Tsuchimatsu *et al.*, 2014). As floral herbivory decreases seed production, it cannot be a directly involved in maintaining flower color polymorphism (Veiga *et al.*, 2015). However, if both pollinators and herbivores prefer the same color, flower color polymorphism may be maintained by antagonistic selective pressure (Frey, 2004, 2007; Strauss and Irwin, 2004; Caruso *et al.*, 2010).

Hepatica nobilis var. *japonica* (Ranunculaceae) harbors remarkable flower color polymorphism, with pink, reddish-purple, purple, bluish-purple, and white flowers, and its flowers bloom in early spring. Its flower is composed of stamens, gynoecium, and colored tepals (a complex of sepals and petals), and blooms upward, but provides no nectar. Our preliminary data (unpublished) suggests that continuous flower color polymorphism could be distinguished using three color types based on the anthocyanin pigment: bluish-purple (containing cyanidin and delphinidin), reddish-purple (containing cyanidin), and white (no anthocyanin). These three flower color types were easily categorized using the DIC color guide (DIC Graphics, Tokyo, Japan). Although Kameoka *et al.* (2017) documented random mating among flowers of different colors (because no neutral genetic differences were found among flower color types), no observation of visiting insect fauna has been conducted. The sister species, *Hepatica americana*, is visited by many flies and solitary bees (Motten, 1982). Because *H. americana* and *H. nobilis* var. *japonica* have similar flower traits, they are expected to be visited by similar insects.

In addition, scars from feeding by herbivores are often found on the tepals of *H. nobilis* var. *japonica* in the field. Flavonoid compounds in plant organs (e.g., leaves and flowers) may confer resistance to herbivores and pathogens (Strauss *et al.*, 2004). Consequently, many plant species store flavonoid components such as anthocyanin in flowers as a defense mechanism (Whittall and Carlson, 2009; Arista *et al.*, 2013),

preventing damage to petals, which can reduce reproductive success (McCall, 2008). Therefore, bluish-purple or reddish-purple flowers may be less affected by herbivores. In this study, to confirm the relationship between three flower color morphs and pollinators and herbivores, insect visitation was observed. To investigate whether the flower color itself is the target of selection, many studies of other plant species with polymorphic flower color have investigated the ability of pollinators to discriminate color morphs (e.g., Gigord *et al.*, 2001; Schemske and Bierzychudek, 2001; Eckhart *et al.*, 2006). Therefore, we also used color recognition analysis (Chittka, 1992; Troje, 1993) to check the visual color discrimination ability of insects visiting flowers (bees and flies). Moreover, the present study aimed to verify the influence on seed production of feeding damage by herbivores, the presence or absence of feeding damage in tepals, stamens, and gynoecium was recorded. The number of seeds was also recorded for each flower color as a measure of fitness.

Materials and Methods

Study species

Hepatica nobilis var. *japonica* (Ranunculaceae) inhabits the forest floor and flowers from early March to April. Because of its remarkable variation in flower (tepal) color and its self-compatibility, this variety has high horticultural value. Consequently, many populations on the Sea of Japan side of central Honshu (Hokuriku area) have been disturbed by illegal removal, whereas there has been less impact on many populations on Sado Island. All of the investigations in the present study were performed on five populations (POP1–5) on Sado Island. We observed pollinators in POP3 and counted damaged flowers and the numbers of seeds in POP1–5. The details of each analysis method are described below.

Pollinator observations

To document visitors to *H. nobilis* var. *japonica*

ica flowers, we conducted daily observations of visiting insects between 10:00 and 16:00 in April 2016 and 2018 in POP3. Flowers of three colors were observed using 24 digital cameras (Optio W30; PENTAX, Tokyo, Japan) as well as visual observation. As the flower of this species closes at night and no visiting insects were found, we made our observations only during the day. For these observations, we randomly selected individual flowers and set up a digital camera to record one photograph every 10s (time-lapse images). Visiting insects were counted and identified using the photographs. We counted the number of flower-visiting insects in the images continuously as one visit. Because the observed numbers of flowers differed between in 2016 and 2018, the data were reported as the visitation ratio (number of visitation/flower/hour).

Assessment of flower color variation

First, flower colors were assigned to color classes using the DIC color guide. This guide provides categorization of many color types, allowing flower color to be easily classified as reddish purple or bluish purple. Next, we selected single flowers from each individual ($n = 85$; 33 bluish purple, 33 reddish purple, and 19 white; Fig. 1a) and measured the percent reflectance of the tepals at wavelengths every 1 nm between 300 and 700 nm using a spectrometer (FLAME-T; Ocean Optics, Dunedin, FL, USA) under a deuterium/halogen light source (DT-MINI-2-GS; Ocean Optics). The analysis was performed using OPWave + (Ocean Photonics, Tokyo, Japan). Because most *Hepatica* flowers are colored in the outer position, we measured the spectral reflectance from the outer position of the tepals. The data were used for color recognition analysis.

Color recognition analysis in insect color space

To determine whether pollinators can discriminate the flower color of *H. nobilis* var. *japonica*, we used the models of Chittka (1992) and Troje (1993) to derive color loci in the color spaces of bees and flies. These models use the spectral sen-

sitivity of honeybee (Chittka, 1992) and blowfly (Troje, 1993; Arnold *et al.*, 2009) receptors to calculate loci. The details of the procedure are described below. First, we calculated P_i , the relative amount of light absorbed by each photoreceptor color type i under mid-day sunlight irradiance:

$$P_i = R_i \int_{300}^{700} S_i(\lambda) I(\lambda) D(\lambda) d\lambda,$$

where $S_i(\lambda)$ is the spectral sensitivity of the receptor classes [ultraviolet (UV), bluish purple, and green], $I(\lambda)$ is the spectral reflectance function of the stimulus, and $D(\lambda)$ is the spectral distribution of the illuminant. The sensitivity factor R_i in the above formula is determined as:

$$R_i = 1 / \int_{300}^{700} S_i(\lambda) IB(\lambda) D(\lambda) d\lambda,$$

where IB is the spectral reflection function of the background to which the receptors adapt. The transformation of photoreceptor absorption (P_i) into receptor excitation (E_i) as physiological input to the brain is given by:

$$E_i = P_i / (P_i + 1),$$

assuming that $E_i = 0.5$ when the photoreceptor absorption (P_i) is the same as the sensitivity factor (R_i) (Chittka, 1992). Then, we estimated the color difference determined by the coding system using a model of each insect type. For bees, we used the model of two unspecified color opponent mechanisms determined by the hexagonal color space (Chittka, 1992). The x and y coordinates were calculated as follows:

$$\begin{aligned} x &= \sin 60^\circ (E_G - E_{UV}) \text{ and} \\ y &= E_B - (E_G + E_{UV})/2. \end{aligned}$$

In this model, the criterion for perceptible color differences in bees was measured using the Euclidean distance. Setting a Euclidean distance over 0.09 as the criterion, this color distance would be required for 60% correct discrimination in the bumblebee *Bombus terrestris* (Dyer, 2006). For flies, a model used to predict the color discriminability of the blowfly *Lucilia* sp. was

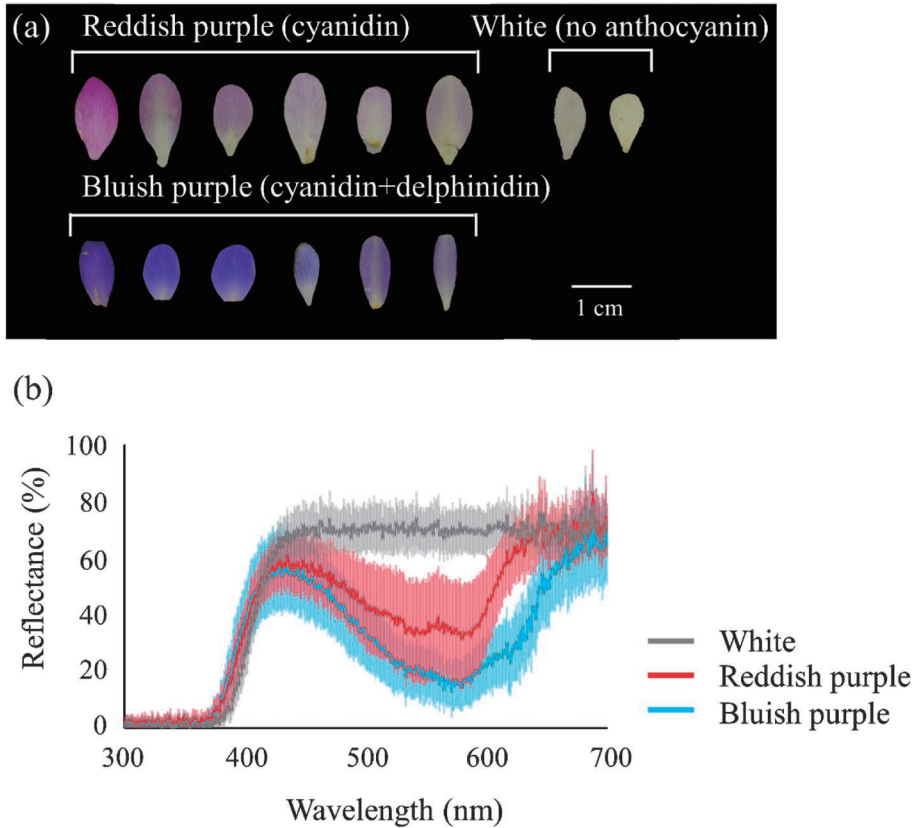


Fig. 1. Flower color variation in *Hepatica nobilis* var. *japonica*. Three flower classes were identified in the field. (a) The three color variants. The visual classification made in the field was consistent with the pigment pattern that was revealed by high-performance liquid chromatography. (b) The average spectral profiles and variation ($\pm$ SE) for each flower color type.

adopted (Troje, 1993; Arnold *et al.*, 2009). This model involves two color opponent mechanisms determined using information for four receptor classes (UV, bluish purple, violet, and green):

$$x = E_{UV} - E_B \text{ and} \\ y = E_V - E_G.$$

The patterns in fly color perception could be explained using four color categories or hues: UV ($x > 0, y > 0$), bluish purple ($x < 0, y > 0$), yellow ($x < 0, y < 0$), and purple ($x > 0, y < 0$) (Troje, 1993).

Evaluation of feeding damage to tepals and its effect on the number of seeds

To assess the feeding damage to tepals, we randomly selected 147 individuals (21 bluish

purple, 53 reddish purple, and 63 white) involved 324 flowers (44 bluish purple, 135 reddish purple, and 145 white) from five populations (POP1–5) at the end of April 2018, and measured reddish purple the presence or absence of feeding damage in tepals, stamens, and gynoecium. We also collected the seed sets from the same flowers in May 2018.

Statistical analysis

The effects of damaged flower organs and number of seeds were tested using a generalized linear mixed model (GLMM) with binomial and Poisson error distributions, respectively. The POP No. and flower colors were used as random variables. We used the R package lme4 to perform the GLMM analyses. Next, Tukey's multi-

ple comparison test was performed to determine whether damaged flower organs and number of seeds differed among flower color types and whether the number of seeds differed between damaged and undamaged flowers, using the multcomp package (Hothorn *et al.*, 2008). All statistical analyses were conducted using R software, ver. 3.2.2 (R Development Core Team, 2015).

Results

Reflectance of flower color

There was substantial variation among the flower color types. The spectral profiles are shown in Fig. 1b. Reddish purple and bluish purple began to peak at two times at 400 nm and 600 nm, whereas white leveled off at 400 nm. In addition, bluish purple began to fall more rapidly than reddish purple at 440 nm.

Pollinator observations and color recognition ability

We obtained a total of 108,135 images, of which 751 depicted visiting insects, for all flower color morphs in April 2016 and 2018 (Table 1). We identified five orders based on the photographs. Based on the photographic evidence, dip-

teran visitors included Syrphidae and Bombyliidae (Fig. 2a, b), and hymenopteran visitors included Andrenidae (Fig. 2c). These insects visited all flower color morphs. Most Coleoptera belonged to Nitidulidae (Fig. 2d), and some belonged to Chrysomelidae (Fig. 2e). Due to inclement weather and low temperatures in 2018, fewer visiting insects were present compared with 2016 (Fig. 3). The photographic evidence indicated that flower-visiting fauna did not differ among flower color morphs; however, the number of visitors differed, especially for Coleoptera. In 2016, the visitation ratio by Coleoptera was markedly higher for white flowers (0.129 in 2016 and 0.04 in 2018) than for bluish-purple (0.033 in 2016 and 0.006 in 2018) and reddish-purple (0.083 in 2016 and 0.002 in 2018) flowers (Fig. 3a, b).

After identifying the visiting insects, we performed color recognition analyses for bees and flies (Fig. 4). Although the number of visiting flowers of Coleoptera was the largest, we didn't perform the color recognition test for Coleoptera because there is no good model. In the analysis of bees, although the three colors were plotted continuously, discrimination between pigmented and white flowers likely occurred in most cases because the color distances between hue catego-

Table 1. Details of the time-lapse photography conducted in this study

Types of flower color	Date	Number of		
		Flowers	Images	Insect visits
Bluish purple	2016/3/31~4/2	21	15,728	46
	2018/4/9~4/11	29	10,149	14
	2018/4/17	12	4,224	8
	2018/4/26	10	4,631	11
Total		72	34,732	79
Reddish purple	2016/3/31~4/2	61	15,701	253
	2018/4/9~4/11	59	11,327	28
	2018/4/17	12	6,640	16
	2018/4/26	28	9,593	26
Total		160	43,261	323
White	2016/3/31~4/2	32	21,135	261
	2018/4/9~4/11	34	5,811	63
	2018/4/17	8	1,026	3
	2018/4/26	10	2,170	0
Total		84	30,142	327

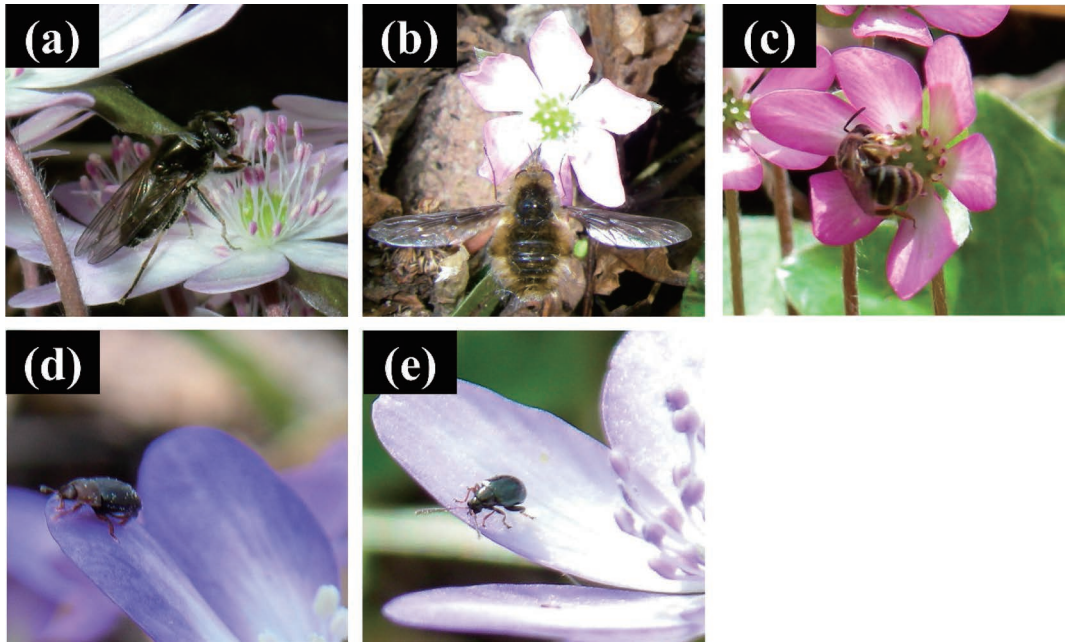


Fig. 2. Selected photographs of visitors to *Hepatica nobilis* var. *japonica* flowers. (a), (b) Diptera (a: Syrphidae, b: Bombyliidae) (c) Hymenoptera (Andrenidae); (d), (e) Coleoptera (a: Nitidulidae, b: Chrysomelidae).

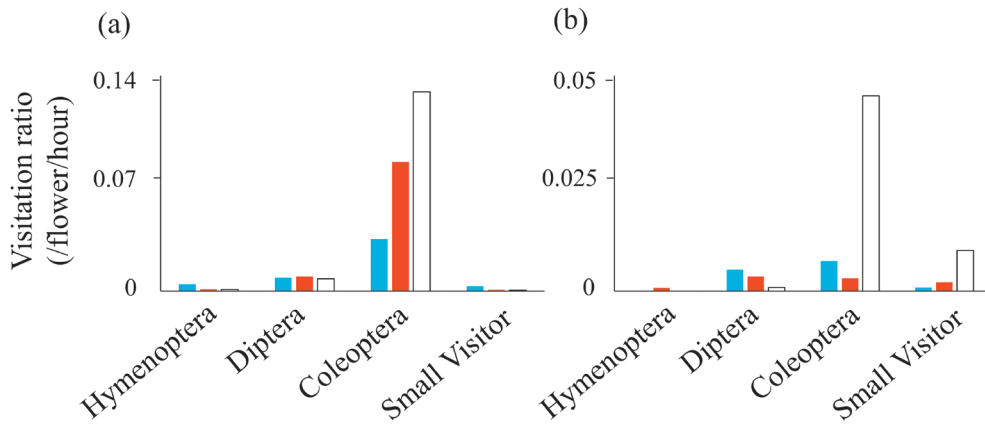


Fig. 3. Insect visitation ratio per flower per hour in (a) 2016 and (b) 2018. The bar colors indicate the bluish-purple, reddish-purple, and white flower color types. The small visitors included Collembola and Araneae.

ries exceeded 0.09 (distance between bluish purple and white = 0.231; distance between reddish purple and white = 0.178, calculated using average of the value of x - and y -axis within each flower color types). Indeed, the distribution of the x -axis values differed significantly between pigmented and white flowers (Tukey's test, $p < 0.001$). On the other hand, the perceptual

color distance between the average loci for bluish purple and reddish purple was 0.0638, and the distribution of values on the x and y axes overlapped (Tukey's test, $p = 0.153$ and 0.163 , respectively). Therefore, these two color types might not be clearly discriminated in the bee color space. A similar pattern was found for the fly color space. All white types were categorized

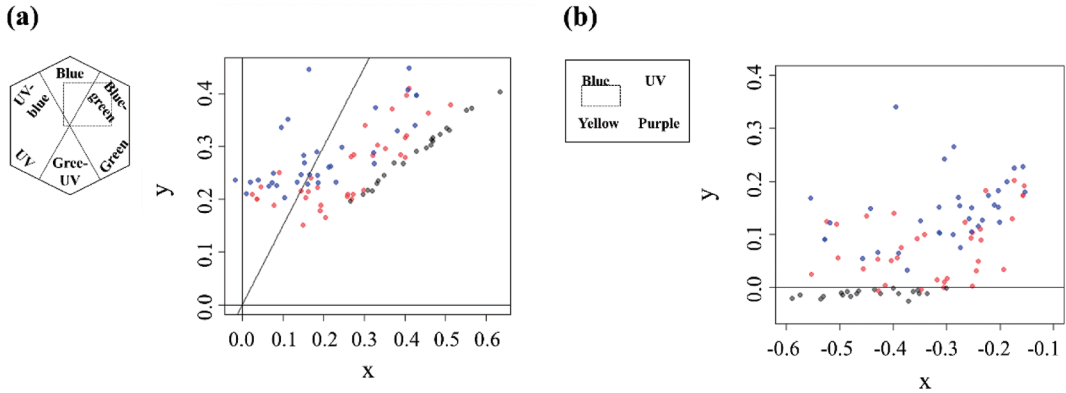


Fig. 4. Corresponding color loci plotted in the color spaces of (a) bees and (b) flies. Plot color shows each flower color type (bluish purple plot: bluish purple; reddish purple plot: reddish purple; black plot: white).

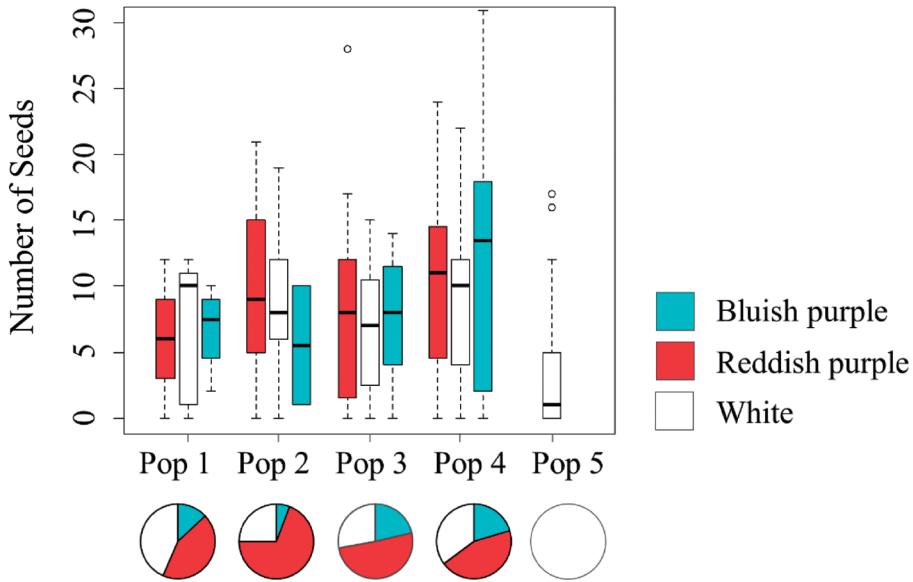


Fig. 5. Numbers of seeds in each flower color types by population. Pie charts below the graph show the flower color frequencies in each population. The data was obtained in 2018.

in the yellow area, whereas most bluish purple and reddish purple types were categorized in the bluish purple area. These results suggest that bees and flies can discriminate between white and pigmented flowers, but cannot clearly discriminate between bluish purple and reddish purple flowers.

Degree of feeding damage and its effect on the number of seeds

Measurements of the seed set and flower color

frequencies for each population revealed that seed production was extremely low in POP5, which consisted only of white flowers (Fig. 5). This may reflect other characteristics of the population (e.g., the population location contains few visiting insects). Therefore, in this study, we excluded the data for the number of seeds for POP5.

GLMM analyses showed that the type of flower color, a fixed term, had significant effects on the damaged ratio for tepals, stamens, and

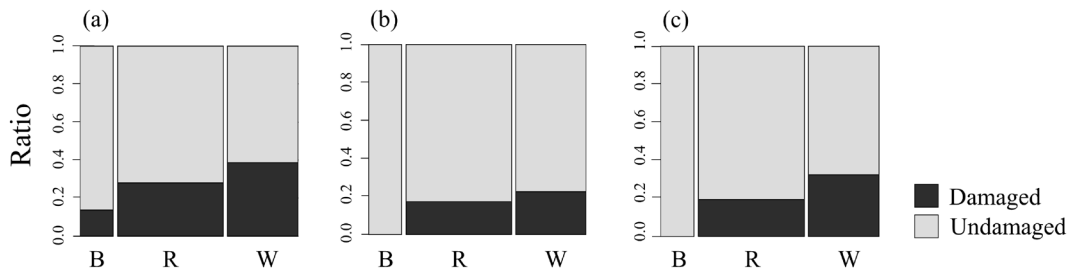


Fig. 6. Damage ratios for (a) tepals, (b) stamens, and (c) gynoecium by flower color type. The widths of boxes represent the numbers of individuals (B: bluish purple, $n = 44$; R: reddish purple, $n = 135$; W: white, $n = 88$).

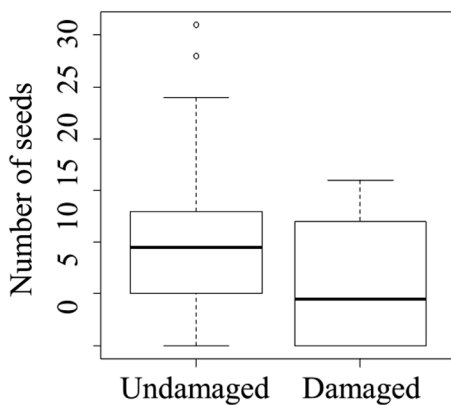


Fig. 7. Numbers of seeds in flowers with damaged and undamaged tepals.

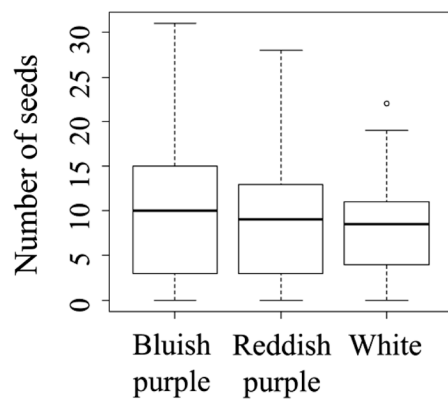


Fig. 8. Numbers of seeds by flower color (bluish purple, $n = 44$; reddish purple, $n = 135$; white, $n = 88$).

gynoecia ($p < 0.01$ for all patterns; POP No. was used as a random variable), and Tukey's multiple comparisons supported the significant ($p < 0.05$) difference in the damaged ratio between bluish-purple and reddish-purple flowers and bluish-purple and white flowers (Fig. 6). Approximately one-third of each of the white and reddish-purple flowers had herbivore damage, whereas less than 10% of the bluish-purple flowers were damaged. As a result, the seed production of damaged individuals was significantly lower than that of undamaged individuals (Tukey's test, $p < 0.001$; Fig. 7; in GLMM analyses, all flower organ damage ratios had a significant effect on the number of seeds, even when flower color was used as the random variable). In addition, no significant difference was seen among flower colors (Tukey's test; $p > 0.05$; Fig. 8).

Discussion

Studies have suggested that flower color polymorphism plays a role as a means of appealing to pollinators (Spaethe *et al.*, 2001; Rausher, 2008). Indeed, the yellow and purple flowers used by Gigord *et al.* (2001) were recognized as different colors by bumblebees. Although a clear difference in the color recognition abilities of Hymenoptera and Diptera was also confirmed for the white flowers and pigmented (i.e., reddish-purple and bluish-purple) flowers of *H. nobilis* var. *japonica*, no difference was found in the visit ratio between white and pigmented flowers (Fig. 3). Although we cannot confirm the pollinators' color preferences based on our results, Kameoka *et al.* (2017) discussed how Hymenoptera and Diptera likely move randomly among flowers of different colors. On the other hand, the number

of flowers visited by Coleoptera was markedly higher for white flowers than for pigmented flowers. Coleoptera seem to innately prefer the white morphs over the pigmented morphs. Some empirical research has been revealed Coleopteran insects' color preference. Several studies have shown that Coleopteran insects [e.g., monkey beetles (Scarabaeidae: *Hopliini*), *Harmonia axyridis* (Coccinellidae), *Meligethes aeneus* (Nitidulidae)] tend to prefer the colors yellow, green, and white rather than blue, red, and orange (Piker and Midgley, 1996; Adedipe and Park, 2010; Döring *et al.*, 2012). Our results are consistent with those findings. However, few studies have examined the color discrimination ability of Coleoptera. Therefore, the color vision of Coleoptera must be elucidated to explore this hypothesis further.

Interestingly, although feeding damage caused a reduction in the number of seeds (Fig. 7a), the average number of seeds per flower did not differ significantly among flower colors, while the damaged ratio was significantly higher in reddish purple and white flower (Fig. 8). From these results, we predict that like Hymenoptera and Diptera, herbivorous also act as pollinators. In this study, although we cannot strongly mention which insect bear the both herbivore and pollinator, from the field observation, Coleoptera is the most likely play that role. Coleopteran insects seem to collect pollen on their bodies when eating tepals, gynoecium and stamens, and then distribute pollen to the gynoecium when moving to nearby flowers. In this case, autogamy would be promoted more in white flowers and reddish purple flowers than in bluish purple flowers. Several studies have shown that the fitness of damaged flowers may be compensated by reproductive gains via male function because autogamy is promoted (Strauss *et al.*, 2001; Avila-Sakar *et al.*, 2003). Indeed, in *Fragaria virginiana* (Rosaceae), increased flower damage led to an increase in selfing, and thereby there is greater opportunities for autogamy (Penet *et al.*, 2008). It is necessary to examine whether the self-pollination rate increases in reddish purple and white *H. nobilis*

var. *japonica* that are visited by Coleoptera, by such means as testing of inbreeding coefficients.

A previous study suggested that the same floral color preference by pollinators and herbivores promotes the maintenance of flower color polymorphism, i.e., pollinators and herbivores cause conflicting selection (Strauss and Whittall, 2007). Our data corroborate that conflicting selection contributes to the maintenance of flower color polymorphism in *H. nobilis* var. *japonica*. Although previous studies have reported that different pollinators and herbivores contribute to conflicting selection (Strauss and Whittall, 2007), our results suggest that the herbivores and pollinators are the same insects, Coleoptera, as discussed above. This is the first report that the same insects act as herbivores and pollinators when involved in the maintenance of flower color polymorphism.

Our study revealed that Coleopteran insects tend to visit white flowers, and it may relate to the promotion of autogamy within a flower. *H. nobilis* var. *japonica* can produce seeds by self-pollination. Although the influence of the negative effects of autogamy has not been reported, negative effects such as inbreeding depression should be evaluated using neutral markers, crossing experiments, and measurements of the germination rates of white and reddish-purple flowers.

Although data were obtained from only 2 years (2016 and 2018), the number of flowers visited by Coleoptera fluctuated throughout this period owing to inclement weather and low temperatures. We predict that this fluctuation may also affect the fitness of each flower. In particular, blue coloration, which is not preferred by Coleoptera, may be advantageous in years when Coleoptera are numerous, because blue flowers incur less feeding damage. On the other hand, white and red colorations, which are preferred by Coleoptera, may be advantageous in years with fewer Coleoptera. Several studies that have focused on other species have shown that such antagonistic and/or fluctuating selection pressures are critical factors in maintaining polymorphisms (Gillespie, 1991; Turelli *et al.*, 2001;

Schemske and Bierzychudek, 2001, 2007). For instance, in two previous studies, four discrete color classes of *Claytonia virginica* were subject to both pollinator-mediated selection for red flowers and opposing directional selection via herbivory and infection by pathogens, factors that fluctuate throughout the year (Frey, 2004; 2007). Further long-term investigations and empirical research on *H. nobilis* var. *japonica* will help shed further light on whether the mechanisms here described contribute to maintaining flower color polymorphism.

Moreover, although we did not investigate the color preferences of Hymenoptera and Diptera, each order has clear differences in their ability to discriminate the colors of the white and pigmented flower found in *H. nobilis* var. *jaoinica*. It is possible that each insect species or individual has color preferences, and that the preferences of both visitors and herbivores may influence the diversity of flower color within a population. At the very least, the heterogeneity of herbivore pressure among flower color morphs would have a relationship with the maintenance mechanism of flower color polymorphism in populations, as damage to tepals significantly influences seed production.

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