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CONSEQUENCES OF DIFFERENCES IN FLOWERING DATE ON SEED PRODUCTION IN HELONIOPSIS ORIENTALIS (LILIACEAE)

Consequences of differences in flowering date on seed production in *Heloniopsis orientalis* (Liliaceae)[1]

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We examined the consequences of differences in flowering date on seed production in the self-compatible herb *Heloniopsis orientalis*. The number of selfed seeds per fruit, as determined by microsatellite markers, did not depend on when the plant flowered, whereas the number of outcrossed seeds per fruit increased with later flowering dates. Consequently, the selfing rate decreased with later flowering dates. The number of seeds (including both selfed and outcrossed ones) per fruit and the seed : ovule ratio increased with later flowering dates. We also examined the effects of pollinators and plant size on seed production. The visitation rate of Diptera did not depend on the flowering season, whereas that of Hymenoptera markedly increased as the flowering season progressed. Diptera stayed longer than Hymenoptera on each plant and flower. Seed production per fruit did not depend on plant size. Thus, the change in selfing rate associated with later flowering dates resulted from the seasonal change in pollinators rather than plant size.

Key words: flowering date; *Heloniopsis orientalis*; Japan; Liliaceae; plant size; pollinator; seed production; selfing rate.

In most angiosperms, flowering date varies among plants within a population, and such variation may result in differences in reproductive output among plants; fruit : flower ratio of a plant (Dieringer, 1991; Ollerton and Lack, 1998; Kelly and Levin, 2000), number of fruits produced by a plant (Ollerton and Lack, 1998; Kelly and Levin, 2000), seed : ovule ratio of a plant (Widén, 1991b), number of seeds produced by a plant (Schmitt, 1983; Widén, 1991b; Kelly and Levin, 2000), and number of seeds per fruit produced by a plant (Dieringer, 1991; Galen and Stanton, 1991; Widén, 1991b) differ among plants within a population depending on their flowering date. The relationships between flowering date and reproductive output are attributed to many endogenous and environmental factors (reviewed in Kelly and Levin, 2000).

In addition, differences in flowering date might affect genetic components of seeds produced by plants; that is, the number of selfed and outcrossed seeds produced might depend on flowering date in self-compatible plants because the pollinator components might change as the flowering season progresses and the flowering date of the plant often depends on plant size. The possibility for xenogamy and geitonogamy depends on the frequency of pollinator visits and the behavioral characteristics of pollinators (de Jong et al., 1993; Kudo, 1993; Snow et al., 1996). Thus, if the pollinator components change over time, the number of selfed and outcrossed seeds might differ among plants with different flowering dates. Also, the selfing rate and flowering date of a plant may be related because both depend on plant size. For example, Kimura et al. (2002) reported that smaller plants produced outcrossed seeds to a greater extent than selfed seed in *Iris gracilipe* and that it may be advantageous for plants to change the proportion of those seeds depending on existing ecological factors, particu-

larly resource status. Thus, we should examine the relationship between the plant size and its selfing rate.

The number of selfed and outcrossed seeds produced by a plant strongly affects fitness of the plant through, for example, inbreeding depression (reviewed in Barrett and Harder, 1996). Hence, in order to elucidate the consequences, selfed and outcrossed seed production needs to be compared among plants with different flowering dates. However, few studies have examined seed production of plants from this viewpoint.

In this study, we examined the effect of flowering date on seed production of the early-spring flowering herb *Heloniopsis orientalis* (Liliaceae). Because the pollinator components change in a short period in early spring (Schemske et al., 1978; Motten, 1986) and fertile plants of *H. orientalis* differ considerably in size within the same population, this species was suitable for our purpose. In this paper, we examine (1) the relationship between flowering date and seed-production characteristics, which included the number of seeds per fruit, seed : ovule ratio, selfing rate, and number of selfed and outcrossed seeds per fruit of the plant using microsatellite markers, (2) whether the change in pollinator components results in a change of characteristics of seed production with flowering date, and (3) whether plant size affects seed-production characteristics.

MATERIALS AND METHODS

Material and study sites—*Heloniopsis orientalis* is a self-compatible perennial herb distributed from an altitude of 0 to 3000 m in Japan and Korea (Kawano and Masuda, 1980). Each fertile plant develops a rosette and a single inflorescence, and 3–14 flowers (average 5–7) bloom in early spring. All flowers of a plant open almost simultaneously and last 7–14 d. The onset of flowering differs by approximately 2 wk among plants in a population. The flowering season of a population is approximately 3–4 wk. The plant is incompletely protogynous, i.e., the stigma has receptivity of pollen on the onset of flowering, which lasts throughout flowering, and the anther dehisces about 2 d after the onset of flowering (Takahashi, 1988). A flower has six stamens and one pistil. The pistil is longer than the stamens, and the stigma separate spatially from the anthers.

The experiments were conducted on Kureha Hill, consisting of >1000 plants (100 m altitude, 36°09′ N, 137°09′ E), in Toyama, Japan, in 2001 and

on Mt. Aoba, consisting of approximately 500 plants (100 m altitude, 38°15′ N, 140°50′ E), in Sendai, Japan, in 2002. The plants at the Kureha Hill site were on the floor of a deciduous forest of *Prunus garayana* and *Castanea crenata*, while those at the Mt. Aoba site were on the floor of a deciduous forest of *Fagus japonica* and *Quercus serrata*. Both sites were sunny during early spring.

Seed production—To examine the dependence of seed production per fruit on the flowering date of the plant, we randomly marked 42 plants on Kureha Hill and 30 plants on Mt. Aoba. We defined the flowering date of a plant as the first flowering date of the plant and recorded the flowering date and the number of flowers for each plant. In order to examine the floral longevity, we defined the end of flowering of a plant as the date when its flowers began to discolor and recorded the duration from the flowering dates to the end of flowering. When fruits had matured, we collected all fruits of each marked plant and counted the number of seeds and unfertilized ovules of each fruit. We calculated the number of seeds per fruit of the plant and the seed : ovule ratio of the plant.

We estimated the selfing rate of these sample plants using microsatellite markers for *H. orientalis* developed by Miyazaki et al. (1999). DNA was extracted with Chelex 100 (SIGMA, St. Louis, Missouri, USA) developed by Walsh et al. (1991). Tissue of a seed or a leaf (0.5 mg) was put in 240 μL 5% Chelex 100 solution and heated in an autoclave at 120°C for 20 min. Extracted DNA was skimmed off the solution and amplified by polymerase chain reaction (PCR) using a thermal cycler (PTC-100TM, MJ Research, Waltham, Massachusetts, USA, in 2001, and iCycler, BIO-RAD, Hercules, California, USA, in 2002). The volume of each reaction mixture was 10 μL and 15 μL and contained approximately 10 ng and 15 ng of template DNA, 0.250 unit (U) and 0.375 U of *Taq* polymerase (Ampli Taq Gold, Applied Biosystems, Foster City, California, USA) in 2001 (Kureha Hill) and 2002 (Mt. Aoba), respectively, and 10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl$_2$, 0.001% (w/v) gelatin, 200 μmol/L of dNTP MIX, and 0.5 μmol/L of primers. An initial denaturation at 94°C for 10 min was prioritized to 35 cycles of denaturation at 94°C for 30 s, followed by annealing for 30 s, extension at 72°C for 1 min, and final incubation at 72°C for 7 min. In this study, three loci (HO68, HO83, HO206) were used with respective annealing temperatures of 48.8°, 49.6°, and 48.9°C (Miyazaki et al., 1999). The PCR products were resolved in 6% denaturing polyacrylamide gel and detected by silver staining.

For each sample plant, DNA was extracted from mother leaf tissue and from 18 randomly selected seeds produced by the plant. We estimated whether seeds were selfed and outcrossed by comparing band patterns of the microsatellite markers. Namely, we determined that a seed was produced by outcrossing if it had a microsatellite marker band(s) not found in that of its mother plant, whereas a seed was judged to have been produced by selfing if all bands at all loci were found in its mother plant. To show the credibility of the selfing rates, paternity exclusion probabilities were also calculated from allele frequencies of loci for both sites using Weir's formulas (Weir, 1996). We calculated the selfing rate of the plant (r) as follows:

$$r = \text{the number of selfed seeds among the 18 seeds examined}/18.$$

From the number of seeds per fruit of the plant, we estimated the number of selfed and outcrossed seeds per fruit of the plant as follows:

The number of selfed seeds per fruit of the plant =
the number of seeds per fruit of the plant $\times$ r.

The number of outcrossed seeds per fruit of the plant =
the number of seeds per fruit of the plant $\times$ $(1 - r)$.

We examined the relationship between the flowering date of a plant and its seed-production characteristics (the number of seeds per fruit, seed : ovule ratio, selfing rate, number of selfed seeds and outcrossed seeds per fruit, and total number of seeds of the plant) with Kendall rank correlation tests. All statistic analyses were conducted using STAT VIEW 5.0 (SAS Institute, Cary, North Carolina, USA).

Pollinator behavior—To examine the effects of pollinators on seed production, we set a 1 m × 1 m quadrat during the flowering season on each observation day at each site. The number of plants growing in the quadrats was 16 on Kureha Hill and three or four on Mt. Aoba. We observed pollinator visits for seven recording cycles of 30-min separated be 30-min intervals from 0900 to 1600 (total of 3.5 h) each day. We also recorded the taxonomic order of pollinators for all visits. One visit was defined as a contact by a pollinator with the stigmas or anthers of a flower. Observations on seasonal changes in pollinator components were done on 27 March, 2 April, and 9 April 2001 on Kureha Hill and on 26 March, 1 April, and 10 April 2002 on Mt. Aoba (all were sunny days). We recorded the number of pollinator visits to the quadrat and calculated the pollinator visitation rate (frequency of pollinator visits per plant per hour). Observations to examine the behavioral characteristics of pollinators on a plant and a flower were conducted on 25 March, 27 March, 1 April, and 2 April 2001 on Kureha Hill and on 26 March, 1 April, and 10 April 2002 on Mt. Aoba (a mixture of sunny and cloudy days). We recorded the number of flowers visited on a plant and the duration of stay on a plant and a flower for each pollinator visit to a plant using a stopwatch (Data Bank 30, Casio, Tokyo, Japan) to the level of 1 s. We examined the differences between the number of flowers visited on a plant as well as the duration of stay on a plant and a flower per visitation by Diptera and those by Hymenoptera with Mann-Whitney U tests.

Effect of plant size—To examine the relationship between flowering date and plant size, we randomly marked 54 plants on Kureha Hill and 47 plants on Mt. Aoba and recorded the flowering dates and the number of flowers for each plant. On Kureha Hill, we dug and collected the whole plant just after the onset of flowering and weighed it after oven drying (60°C for 72 h) to the level of 1 mg. Since we obtained a positive correlation between the number of flowers of a plant and its plant dry mass (Kendall rank correlation test: $N = 54$, $r^2 = 0.7081$, $\tau = 0.684$, $P < 0.0001$), we used the number of flowers as an index of plant size. We examined the relationships between the indexes of plant size (the number of flowers of a plant and the plant dry mass) and its flowering date and between the number of flowers of a plant and its seed-production characteristics (the number of seeds per fruit, seed : ovule ratio, selfing rate, number of selfed seeds and outcrossed seeds per fruit, and total number of seeds of the plant) with Kendall rank correlation tests.

Bagging treatment—To examine whether autogamy occurs, we bagged randomly chosen plants ($N = 17$ on Kureha Hill and $N = 20$ on Mt. Aoba). We recorded the flowering dates and bagged the plants before flowering to prevent natural pollination. When fruits had matured, we collected all fruits of each marked plant and counted the number of seeds and unfertilized ovules of each fruit. We calculated the seed : ovule ratio of the bagged plants. We examined whether arcsine seed : ovule ratio of the bagged plants differed from zero with a t test.

RESULTS

Consequences of microsatellite markers analysis—Three microsatellite makers were variable, i.e., the number of alleles at each locus was five on Kureha Hill and six or seven on Mt. Aoba (Table 1). With all markers combined, the paternity exclusion probability was high (0.9059 on Kureha Hill, 0.9492 on Mt. Aoba). Hence, these markers were sufficiently informative for analysis of selfing rate.

Dependence of seed production on flowering date—The number of seeds per fruit and the seed : ovule ratio of the plant increased with later flowering dates ($N = 42$, $\tau = 0.445$, $P < 0.0001$ on Kureha Hill, and $N = 30$, $\tau = 0.258$, $P = 0.0450$ on Mt. Aoba for the number of seeds per fruit of the plant; $N = 42$, $\tau = 0.395$, $P = 0.0002$ on Kureha Hill, and $N = 30$, $\tau = 0.373$, $P = 0.0040$ on Mt. Aoba for the seed : ovule ratio of the plant). The selfing rate of the plant decreased with later

TABLE 1. Number of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E), and estimated paternity exclusion probability (PEP) at three microsatellite loci.

| | Kureha 2001 ($N = 42$) | | | | Aoba 2002 ($N = 30$) | | | |
Locus	Allele	H_O	H_E	PEP	Allele	H_O	H_E	PEP
HO68	5	0.825	0.759	0.5274	7	0.830	0.800	0.6562
HO83	5	0.754	0.768	0.5442	6	0.767	0.803	0.6115
HO206	5	0.680	0.780	0.5631	7	0.800	0.730	0.6196
Total	15			0.9059	19			0.9492

flowering dates (Fig. 1). Consequently, the number of selfed seeds per fruit did not depend on flowering date of the plant (mean ± 1 SE: 151.60 ± 5.03 in 23–26 March, 139.25 ± 3.06 in 27 March–1 April, and 129.79 ± 7.98 in 2–6 April on Kureha Hill, and 143.49 ± 5.97 in 23–29 March, 171.62 ± 4.03 in 30 March–2 April, and 127.99 ± 12.17 in 3–8 April on Mt. Aoba), but that of outcrossed seeds per fruit increased with later flowering dates (98.01 ± 2.63 in 23–26 March, 137.74 ± 3.57 in 27 March–1 April, and 183.27 ± 13.07 in 2–6 April on Kureha Hill, and 124.48 ± 9.45 in 23–29 March, 184.23 ± 5.80 in 30 March–2 April, and 210.73 ± 9.62 in 3–8 April on Mt. Aoba) (Fig. 2). The total number of seeds of a plant increased with later flowering dates on Kureha Hill ($N = 42$, $\tau = 0.280$, $P = 0.0297$), but did not depend on flowering date of the plant on Mt. Aoba ($N = 30$, $\tau = 0.136$, $P = 0.2051$). Also, early-flowering plants had longer floral longevity than late-flowering ones ($N = 42$, $\tau = -0.747$, $P < 0.0001$ on Kureha Hill and $N = 30$, $\tau = -0.903$, $P < 0.0001$ on Mt. Aoba).

Pollinator behavior—Visitation rate (frequency of pollinator visits per plant per hour) of Diptera did not depend on flowering date, whereas that of Hymenoptera markedly increased as the flowering season progressed (Fig. 3). Diptera were flies and syrphid flies, and most Hymenoptera were solitary bees (*Andrena* spp. and *Lasioglossum* spp.). The number of flowers visited on a plant per visitation did not differ between Diptera and Hymenoptera (Fig. 4). However, Diptera stayed longer on a plant and on a flower per visitation than did Hymenoptera (Fig. 5).

Effect of plant size—Neither plant dry mass nor the number of flowers correlated with flowering date (Table 2). The number of flowers, which was an index of plant size, did not correlate with seed-production characteristics, i.e., the number of seeds per fruit, seed : ovule ratio, selfing rate, and number of selfed or outcrossed seeds per fruit of the plant (Table 3). On the other hand, the plants that had larger numbers of flowers had larger numbers of seeds ($N = 42$, $\tau = 0.452$, $P < 0.0001$ on Kureha Hill, and $N = 30$, $\tau = 0.537$, $P < 0.0001$ on Mt. Aoba).

Bagging treatment—The seed : ovule ratios of the bagged plants were 0.006 ± 0.004 (mean ± SE) on Kureha Hill and 0.008 ± 0.005 on Mt. Aoba. These results did not differ statistically from zero ($N = 17$, $t = 1.384$, $P = 0.1835$ on Kureha
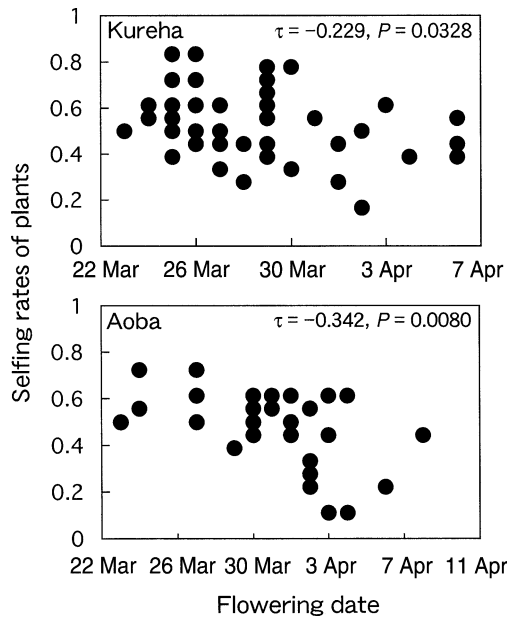

Fig. 1. Relationship between flowering dates and selfing rates of plants on Kureha Hill ($N = 42$) and Mt. Aoba ($N = 30$). τ is the Kendall rank correlation coefficient.
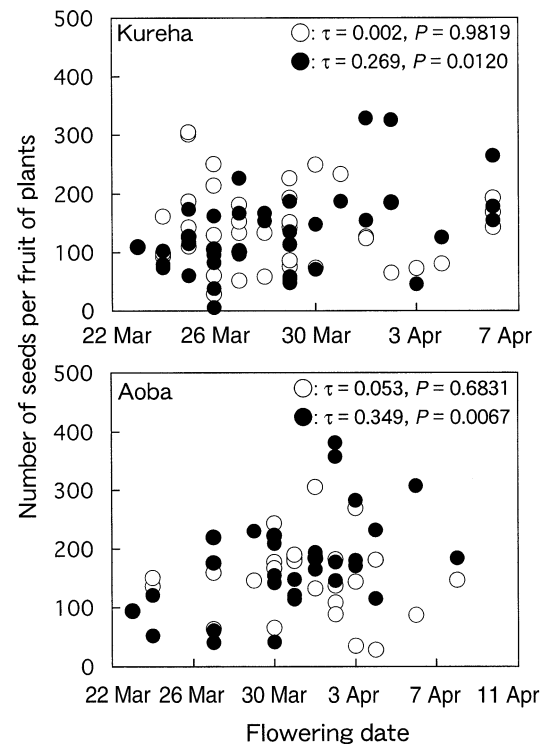

Fig. 2. Relationship between flowering dates and the number of selfed (open circles) and outcrossed (closed circles) seeds per fruit of plants on Kureha Hill ($N = 42$) and Mt. Aoba ($N = 30$). τ is the Kendall rank correlation coefficient.
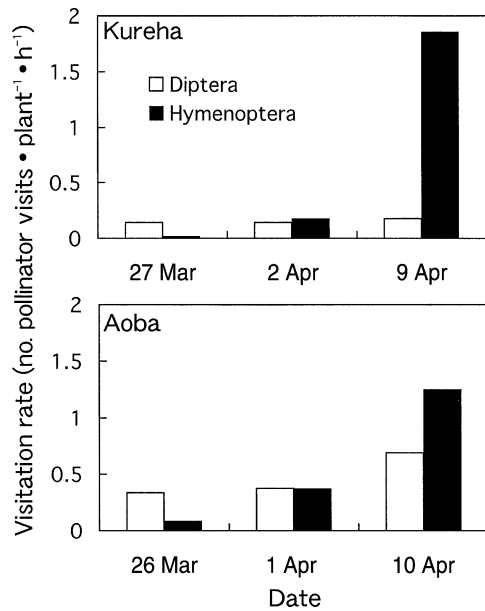
Fig. 3. Pollinator visitation rate during the *Heloniopsis orientalis* flowering season.
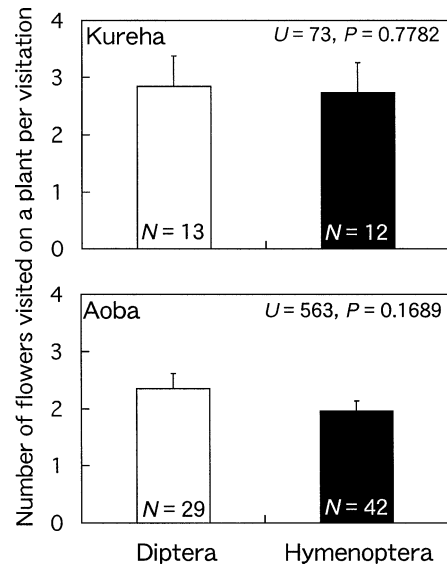

Fig. 4. Number of flowers visited on a plant per visitation by Diptera and Hymenoptera (means + 1 SE). Differences between the number of flowers visited on a plant per visitation by Diptera and by Hymenoptera were examined with Mann-Whitney U tests.

Hill, and $N = 20$, $t = 1.720$, $P = 0.1016$ on Mt. Aoba). Thus, autogamy was negligible in these populations.

DISCUSSION

Dependence of seed production on flowering date—In *H. orientalis*, which is a self-compatible plant, the genetic components of seeds changed with flowering date. The selfing rate decreased with later flowering dates of the plants (Fig. 1), while the number of outcrossed seeds per fruit increased (Fig. 2). The number of seeds (including both selfed and outcrossed ones) per fruit and the seed : ovule ratios also increased with later flowering dates. The total number of seeds of a plant increased with later flowering dates on Kureha Hill, but did not depend on flowering date of the plant on Mt. Aoba. Thus, the later-flowering plants may have higher female fitness due to the avoidance of inbreeding depression because they had low selfing rates, but that has not been determined for this species or these populations. In previous studies (Dieringer, 1991; Widén, 1991b; Totland, 1994; Ollerton and Lack, 1998; Kelly and Levin, 2000), the number of seeds and fruits produced by a plant has been shown to depend on its flowering date. However, as the study plants were self-incompatible in those studies, it was not examined whether the selfing rate of the plant also depended on its flowering date. In the present study, the selfing rate was found to decrease with later flowering date in *H. orientalis*. Further studies on other self-compatible species will be necessary to examine whether this trend is general.

Proximate factors affecting genetic components of seeds—*Effect of pollinators*—In this study, the number of flowers visited on a plant per visitation did not differ between Diptera and Hymenoptera (Fig. 4). However, Diptera stayed longer than Hymenoptera on each plant and flower per visitation (Fig. 5). Because Diptera use flowers not only for food but also for mating and basking (Kudo, 1995; Johnson and Midgley, 1997; Johnson and Dafni, 1998), they stay longer on plants and flow-

ers and fly a shorter distance among plants and flowers (Yumoto, 1986; Totland, 1994). On the other hand, because Hymenoptera use flowers mainly for food, namely, pollen and nectar, they move actively in terms of pollination among flowers and plants. Diptera might have a greater chance of inducing within-flower selfing because they stay longer on flowers than Hymenoptera. Furthermore, Diptera might have a greater chance of inducing geitonogamy because Diptera might have a greater chance of pollen carryover among the flowers on a plant due to their longer stay on flowers. Thus, Hymenoptera may be more active in terms of pollination than Diptera.
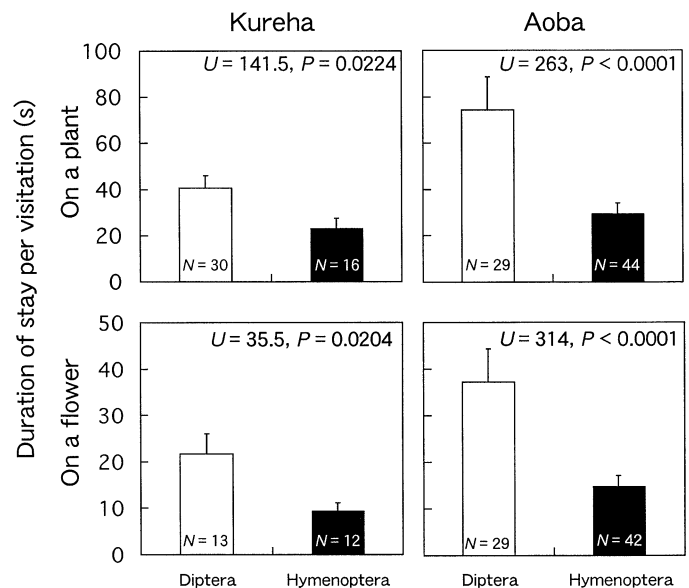

Fig. 5. Duration of stay on a plant and a flower per visitation by Diptera and Hymenoptera (means + 1 SE). Differences between duration of stay on a plant and a flower per visitation by Diptera and by Hymenoptera were examined with Mann-Whitney U tests.

TABLE 2. Relationship between flowering date and indexes of plant size (Kendall rank correlation test).

Index of plant size	Kureha 2001 (N = 54)			Aoba 2002 (N = 47)		
	Average ± SD	τ	P	Average ± SD	τ	P
Plant dry mass (mg)	5849 ± 2627	−0.055	0.5606	—		
No. flowers	6.54 ± 1.78	0.078	0.4078	4.49 ± 1.37	0.118	0.2409

Furthermore, the frequency of Hymenopteran visits increased later in the flowering season, whereas that of Diptera did not change during the flowering season (Fig. 3). This change can explain the change in seed production; the number of outcrossed seeds per fruit increased in the plants that flowered later, whereas that of selfed seeds per fruit did not change with the flowering date (Fig. 2). Although the peak of the density of flowering plants was in the middle of flowering season in both sites, the selfing rate increased with later flowering dates. Hence, we presume that the seasonal change in pollinators rather than those of density in potential mates affected seed production. As autogamy was negligible, almost all seeds were considered to be fertilized by pollinators. Thus, the differences in the behavioral characteristics between Hymenoptera and Diptera and the seasonal change of pollinators might result in changes in the number of selfed and outcrossed seeds with the flowering date.

Effect of plant size—Seed production and flowering date of a plant may be related because both depend on plant size, as has been reported in some species (Dieringer, 1991; Widén, 1991b; Kelly, 1992; Ollerton and Lack, 1998; Kelly and Levin, 2000; Wada and Uemura, 2000; but see Schmitt, 1983). For example, Widén (1991b) found that in *Senecio integrifolius*, larger plants flowered later and produced fewer seeds per fruit than smaller ones. However, in this study, the plant dry mass and the number of flowers on the plant—indexes of plant size—were not correlated with flowering date (Table 2). Neither did the number of flowers on the plant correlate with number of seeds per fruit, the seed : ovule ratio, the selfing rate, and the number of selfed or outcrossed seeds per fruit of the plant (Table 3). Thus, the relationship between flowering date and seed production per fruit in *H. orientalis* cannot be explained by differences in plant size.

Conclusion—We found that not only the number of seeds but also the genetic component of seeds changed with flowering date in the self-compatible herb *H. orientalis*. The selfing rate decreased with later flowering dates of the plants, and plants that flowered later might gain greater female fitness.

Why then were there early-flowering plants in spite of the disadvantage in terms of female fitness? We consider two possibilities: the flowering date of a plant is a heritable charac-

teristic (e.g., Pors and Werner, 1989; Fox, 1990; Widén, 1991a; Mitchell-Olds, 1996) or it is not (e.g., Kelly and Levin, 1997). In the former case, the early-flowering plants might gain fitness as pollen donors. For example, as this species is incompletely protogynous (Takahashi, 1988), the early-flowering plants might gain greater fitness as pollen donors (e.g., Molau, 1991; Wada and Uemura, 2000). Moreover, as early-flowering plants had longer floral longevity than the late-flowering ones, the early ones might have more pollen-flow opportunities than late ones in spite of low pollinator activities. Thus, the early-flowering plants might compensate for the disadvantage in female fitness by gaining greater male fitness. Future studies might address the effect of flowering date on male reproductive success using microsatellite markers for paternity analysis. Here, we should also examine the magnitude of inbreeding depression of this species to examine whether late-flowering plants avoided inbreeding depression because they had low selfing rates. In the latter case, however, the flowering date might depend on the microhabitats of plants or be determined by chance. For example, because the time of snow melt varies among microhabitats of the same population, microhabitat characteristics might account for the difference in flowering date among plants. We should also examine the heredity of flowering date using quantitative genetics; ramets should be grown in different habitats and compared as to flowering dates. Such investigations are needed to answer these questions.

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TABLE 3. Relationship between the number of flowers of a plant and seed production (Kendall rank correlation test).

Seed production (of the plant)	Kureha 2001 (N = 42)		Aoba 2002 (N = 30)	
	τ	P	τ	P
Number of seeds per fruit	0.147	0.1690	0.148	0.2509
Seed : ovule ratio	0.035	0.7466	0.159	0.2169
Selfing rate	0.019	0.8607	−0.107	0.4053
No. selfed seeds per fruit	0.132	0.2180	−0.042	0.7452
No. outcrossed seeds per fruit	0.112	0.2981	0.215	0.0953

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