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Author(s)	Kawai, Yuka; Kudo, Gaku
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Instructions for use

Local differentiation of flowering phenology in an alpine-snowbed herb *Gentiana nipponica*

Kawai Yuka and Kudo Gaku

Graduate School of Environmental Earth Science,

Hokkaido University, 060-0810, Sapporo, Japan

e-mail: kawawawa@ees.hokudai.ac.jp

Fax: +81-11-7064954

Abstract

Phenological events of alpine plants are strongly influenced by the seasonal thermal conditions at the local scale that are caused by heterogeneity in snowmelt time. Populations in late-snowmelt locations suffer from a short period for seed maturation, in which rapid flowering that occurs soon after snowmelt (i.e., a low thermal requirement for flowering) is advantageous. To test the possibility of local adaptation of flowering phenology, we compared reproductive phenology, the preflowering period, and the thermal requirement for flowering of the alpine-snowbed species Gentiana nipponica Maxim. between populations inhabiting early- and late-snowmelt sites within a local area. Plants in the late-snowmelt population showed a shorter preflowering period than in the early-snowmelt population; nevertheless, they often failed to set fruit owing to the short growth period. To test the plasticity and genetic regulation of flowering phenology, we performed a reciprocal transplant experiment between early- and late-snowmelt populations. Although the preflowering period showed clear plastic responses depending on the transplanted habitats, the lower thermal requirement for flowering was retained in plants originating from the late-snowmelt populations, even after transplantation. Therefore, habitat-specific differentiation of flowering phenology was genetically determined between these local populations.

Introduction

Variation in flowering time the results from environmental heterogeneity and (or)

genetic variation (Hall and Wills 2006) is a critical issue determines the reproductive

success of plants (e.g., Rathcke and Lacey 1985; Primack 1987; Thórhallsdóttir 1998;

Sandring et al. 2007). Because the optimal timing of flowering may vary across

different habitats, natural selection may act on flowering phenology to maximize

reproductive success under particular abiotic and biotic environments. In higher

latitude and altitude ecosystems, where the cool climate restricts the length of the

growth season, phenological events should be strongly influenced by the seasonal

changes in thermal conditions at a local scale (Rathcke and Lacey 1985; Fitter et al.

1995; Kudo and Suzuki 1999).

In alpine environments, the growing season is cool, short, and curtailed at both

ends by subzero temperatures or snow cover. Such temporal variation in snowmelt

pattern determines the timing of flowering and the available growth period of plants

(Bliss 1971; Galen and Stanton 1991; Kudo 1991; Wipf et al. 2006). Too-early flowering

may reduce reproductive success if the risk of frost damage increases (Inouye 2008) or

pollinator service is insufficient early in the season (McCall and Primack 1992; Kudo

1993; Totland 1994; Kameyama and Kudo 2009). On the other hand, too-late flowering

may result in the failure of seed maturation owing to the short growth period (Primack

1987; Kudo 1991, Thórhallsdóttir 1998). Therefore, the direction of selective forces on

optimal flowering time should vary among habitats with different snowmelt times.

In an ecosystem with a clear temperature seasonality, cumulative temperature

from the beginning of the growth season is a cue for determining the time of flowering

initiation (Hülber et al. 2010). The cumulative temperature above a certain threshold

value (growing degree day, GDD) has been used as an index of flowering traits for

temperate, subalpine, alpine, and arctic plants in previous studies (e.g., Kudo and

Suzuki 1999; Molau et al. 2005; Wipf 2010; Hülber et al. 2010). The thermal requirement for the onset of flowering is usually specific to individual species. However, previous studies demonstrating the intraspecific variation in flowering properties are mostly restricted in the comparisons at the geographic scale, such as latitudinal and altitudinal gradients (Stinchcombe et al. 2004; Wagner and Simons 2009).

Divergent selective forces that reflect environmental heterogeneity may cause local adaptation of phenology and other life-history traits between populations along complex environmental gradients (Hall and Willis 2006; Wagner and Simons 2009). Furthermore, genetic isolation between populations is required to fix specific traits within populations (Sambatti and Rice 2006). Because flowering time and period of alpine plants are strongly influenced by the time of snowmelt, gene flow via pollination process may be restricted between neighboring populations that inhabit different snowmelt sites owing to phenological isolation (Hirao and Kudo 2004). Therefore, alpine plant species with a wide distribution range along the snowmelt gradient should be a good material for the study of local adaptation of flowering phenology (Kudo and Hirao 2006). First, rapid flowering after snowmelt may be disadvantageous in early-snowmelt populations, while it may be advantageous in late-snowmelt populations, as described previously. Therefore, the optimal flowering schedule may be different between populations that inhabit different snowmelt conditions. Second, the flowering period of individual populations strongly depends on the time of snowmelt, and this may cause a temporal separation of flowering season between adjacent populations (Kudo 1991), resulting in genetic isolation via pollen dynamics (Hirao and Kudo 2008). To examine the extent and direction of selection on flowering traits along the snowmelt gradient, comparisons of thermal requirement for flowering and reproductive output among populations are most desirable.

In this study, we compared reproductive phenology (flowering and fruiting times) and the thermal requirement for flowering of the alpine-snowbed herbaceous species *Gentiana nipponica* Maxim, among populations inhabiting different snowmelt conditions. This species is distributed widely along the range of the snowmelt gradient, and the flowering season of individual populations depends strongly on snowmelt time (Kudo and Suzuki 1999; Hirao and Kudo 2004). *Gentiana nipponica* is a late-flowering species in snowbed plant communities (Kudo 1991), populations in late-snowmelt habitats suffer especially from the short period for seed maturation. It is assumed that rapid flowering after snowmelt (because of a lower thermal requirement for flowering) is required in late-snowmelt populations if flowering progress is under the regulation of temperature. In addition, a previous study (Hirao and Kudo 2004) revealed the existence of spatial genetic structure in this species among adjacent populations within a local area, in which genetic distance between populations was more related to phenological isolation rather than to simple geographic distance. This implies the possibility of local adaptation to habitat-specific environments along the snowmelt gradient.

The objective of this study is to answer the following questions.

(1) Does the variation in flowering time of *G. nipponica* influence the fruit-set success of individual populations inhabiting areas with different snowmelt conditions?

(2) Is the preflowering period shorter in late-snowmelt populations? If so, is it explained by the difference in thermal requirement for flowering between populations?

(3) Does the variation in thermal requirement reflect genetic variation between populations or simply the plastic response to each habitat?

To clarify whether the variation in flowering phenology of *G. nipponica* is expressed by plasticity or genetic regulation, we performed a reciprocal transplant experiment between early- and late-snowmelt populations. If the thermal requirement for flowering

is genetically determined in each population, it would suggest the existence of local differentiation of flowering phenology to specific snow conditions.

Materials and Methods

Study site

This study was conducted in the central part of the Taisetsu Mountains, Hokkaido, northern Japan (43°33'N, 142°51'–52'E). Because of heavy snowfall and the prevailing northwest winds during winter, deep snowdrifts are established on southeastern-facing slopes that cause a clear gradient of snowmelt time within a local area. A longterm observation of snowmelt pattern in this area revealed that the time of snowmelt varied greatly from year to year, but the spatial snowmelt pattern was consistent every year (Kudo and Hirao 2006), indicating the ecological importance of snowmelt gradients in this alpine ecosystem. The timing of snow disappearance ranges from early June to mid-August. Snowfall begins in late September, and the ground is covered with snow by early October (Hirao and Kudo 2004; Kudo and Hirao 2006).

   We selected three measurement sites: two early-snowmelt plots (E-1 and E-2) and one late snowmelt plot (L). Plot E-1 was located at 1890 m elevation on a southeast slope of Mt. Kaun. Plot E-2 was located at 1840 m altitude and 2 km east of plot E-1. In the early-snowmelt plots, snow usually disappeared from early to late June, and the flowering peak of most plants were in late July to early August. Plot L was located at 1800 m altitude and 250 m southwest of plot E-2, where snow usually disappeared from late July to early August, and the flowering peak was around late August (see Fig. 1). In our experimental system, therefore, the effect of elevation may be negligible. Dwarf shrubs, such as *Siversia pentapetala* Greene, *Rhododendron aureum* Georgi, and *Phyllodoce caerulea* (L.) Babington, are dominant in the early-snowmelt habitat, while

herbaceous species, such as *Primula cuneifolia* Ledeb., *Potentilla matusmurae* Th. Wolf, and *Peucedanum multivittatum* Maxim., are common in the late snowmelt habitat in this region (Kudo and Suzuki 1999).

Study plant

Gentiana nipponica Maxim. (Gentianaceae) is an evergreen perennial herb that is widely distributed in alpine meadows and snowbeds of mountain regions in Japan. *Gentiana nipponica* commonly forms a small clump of several prostrate stems. The plant height is < 10 cm, and the diameter of clumps ranges from a few centimeters to 15 cm. Individual stems grow continuously over several years, and evergreen leaves accumulate on these stems without senescence. When the leaf number reaches a critical level, flowering occurs (Y. Kawai and G. Kudo, personal observation). *Gentiana nipponica* has a determinate inflorescence in which total flower number has been fixed in the previous year. Reproductive stems typically have one to five flowers in a simple terminal or axillary cymes that die back soon after seed production. The terminal flowers open first and axillary and lower positioned flowers open subsequently within inflorescences. Although this species can set fruit by self-pollination, seed production by autogamous self-pollinating is rare owing to the dichogamous flowering. Bumblebees are major pollinators, but dipteran and lepidopteran insects also visit this species.

Flowering phenology

We monitored the general pattern of flowering and fruiting phenologies in each plot at 5–10 d intervals from 2005 to 2009. The reproductive stage was categorized by visual observation in each plot as flowering initiation (1%– 25% plants opening flowers), peak flowering (about 25%–75% plants opening), late flowering (>75% plants finished flowering), developing fruits (fruiting but no seed dispersal), and fruit maturation (seed

dispersal). For the analysis of general trends, we compared the first observation day of snowmelt with the day of flowering occurrence among plots.

To measure the first flowering day (FFD) of individual plants, we conducted detailed measurements of flowering occurrence within a 3 m × 3 m quadrat in each plot. In 2005, we randomly selected 46 reproductive stems with floral buds in plot E-1 and 31 reproductive stems in plot E-2 from different clumps within the quadrat before the flowering season. In 2006, 50 reproductive stems in plot E-1 and 100 reproductive stems in plot L were selected within the quadrats. All selected stems were tagged for identification before flowering. Previous studies reported that flowering phenology is sometimes influenced by total flower production (i.e., Ollerton and Lack 1998), the number of flowers per inflorescence was recorded for every stem. We observed the flowering conditions of individual flowers every 1-2 d until all labeled flowers opened. The date the terminal flower opened was used as the FFD.

Transplant experiment

To examine the response of flowering phenology to different snowmelt conditions, a transplant experiment was performed between early- and late- snowmelt populations (plots E-2 and L). In late September 2005, 35 individuals of similar clump size were carefully excavated in each plot. Twenty individuals were transplanted to the other plot, from plot E-2 to plot L (E to L) and from plot L to plot E-2 (L to E). The remaining 15 individuals were replanted in the same plot to serve as controls (E to E and L to L). Then, FFD for each reproductive stem within the clumps was measured in 2006 and 2007, as mentioned above.

Effective cumulative temperature

The snowmelt day in each plot was determined by the soil surface temperature, which was measured by automatic loggers (StowAway Tidbit, Onset Co., USA) that were set in the central part of each plot. Temperature was recorded at 2-h intervals during the experimental period. Effective cumulative temperature above a specific threshold value was used to describe the phenological progress of arctic and alpine plants (Thórhallsdóttir 1998; Wipf et al. 2006; Hülber et al. 2010). Growing degree days (GDD) between snowmelt and the FFD were calculated as the sum of daily mean temperatures at soil surface using a threshold value of 5ºC (Kudo and Suzuki 1999). Because of a deficiency of data from plot E-2 in 2005 owing to equipment failure, we used the temperature record for plot E-1 for the analysis of plot E-2 for 2005. We confirmed that there was little difference in soil surface temperature during the snow-free period between the plots.

Statistical analyses

Statistical analyses were conducted using R statistical software (version 2.9.0, R Development Core Team 2009). The time of snowmelt and flowering initiation were tested over 5 years using a generalized linear model (GLM) and Poisson error distribution, in which plot (E-1, E-2, or L) and year (2005–2009) were considered as explanatory variables. In the analyses of preflowering period after snowmelt and the thermal requirement for FFD (expressed as GDD), we applied a linear mixed-effects model (LME; Pinheiro and Bates 2000) with restricted maximum likelihood estimates, in which plot (E-1, E-2, or L) and flower number per inflorescence (representative of size effect) were considered as explanatory variables and year (2005 or 2006) was considered as a random factor. For all of these analyses, the best-fitting variables were selected based on Akaike's information criterion (AIC), in which the best model showed the lowest AIC values (Burnham and Anderson 2002).

For the transplant experiment, we employed LME to analyze the dependence of preflowering period and GDD on the original habitat (genetic effect) and transplanted habitat (environmental effect). In this model, the original plot and transplanted plot (E-2 or L) were considered as fixed variables, including their interaction and clump (individual plant) as a random variable nested in each plot.

Results

Flowering phenology

Variations in snowmelt time and reproductive phenology in individual plots over 5 years (2005–2009) are shown in Fig. 1. The average snowmelt date was June 4 ± 6.0 d (Mean ± SD) in plot E-1, June 19 ± 6.1 d in plot E-2, and July 30 ± 12.1 d in plot L. In the GLM analysis, the difference in snowmelt time between plots E-1 and E-2 was marginal ($z = 1.96$, $P = 0.050$), while the difference between plots E-1 and L was highly significant ($z = 6.52$, $P < 0.0001$). Variation in snowmelt date was especially large in plot L in comparison with plots E-1 and E-2. For example, snowmelt in plot L occurred almost 1 month earlier in 2008 in comparison with 2009, while difference between years was less than 10 d in plots E-1 and E-2.

The period from snowmelt to flowering was 40–57 d in plot E-1, 41–50 d in plot E-2, and 27–34 d in plot L. Flowering initiation showed remarkable variations among years and plots (E-1, July 20 ± 4.6 d; E-2, August 1 ± 5. 9 d; L, August 25 ± 14.0 d), and the yearly variation in plot L was larger compared with plots E-1 and E-2. The GLM revealed that there was no significant difference in the time of flowering initiation between plots E-1 and E-2 ($z = 1.32$, $P = 0.19$), while that in plot L was significantly later ($z = 3.63$, $P < 0.001$). Fruit maturation started about 1 week after flowering in each population. Because snowmelt in 2005, 2007, and 2009 was late, plants in plot L

failed to set fruit owing to the short growth season and snowfall before fruit maturation. Especially in 2009, no plants in plot L produced flowers because of the insufficient growth period and cool temperature during the summer.

Detailed comparison of flowering phenology was conducted using the data from 3 m × 3 m quadrats in 2005 and 2006 (Table 1). The preflowering period after snowmelt was significantly shorter in plot E-2 than in plot E-1 in 2005 ($t = -6.89$, $P < 0.0001$) and shorter in plot L than in plot E-1 in 2006 ($t = -44.55$, $P < 0.0001$). Furthermore, plants with larger inflorescences tended to open flowers earlier ($t = -2.78$, $P = 0.006$). There was a significant difference in the thermal requirement for flowering between plots E-1 and L in 2006 ($t = -10.06$, $P < 0.0001$), while there was no significant difference between plots E-1 and E-2 in 2005 ($t = 1.62$, $P = 0.11$). Plants with larger inflorescences tended to have a lower thermal requirement for flowering ($t = -2.50$, $P = 0.013$).

Transplant experiment

Survival rates of transplanted clumps (E to E, L to E, E to L and L to L) were relatively high until the next flowering season (2006), ranging from 73% (E to E) to100% (L to L). However, because survival rates were low and there were no flowering stems in the L to E plants in 2007, we used 2006 data for the statistical analysis of flowering phenology.

The preflowering period after snowmelt differed significantly among treatments in 2006. Plants that originated from plot L bloomed significantly faster than plants that originated from plot E-2 in both transplanted sites (Table 2, Fig.2). LME analysis revealed that there were significant effects from the original plot L ($t = -3.38$, $P = 0.0017$) and the transplanted plot L ($t = -11.53$, $P < 0.0001$) on the preflowering period, while the interaction effect between them was not significant ($t = -0.70$, $P = 0.49$). This trend remained also in 2007, although there was no flowering in the L to E plants

(Table 2). This result indicated that the actual flowering schedule of *G. nipponica* was affected by both genetic and environmental factors. Furthermore, the GDD for FFD was lower in plants that originated from plot L than plants originated from plot E-2, irrespective of transplant site (Table 2, Fig. 2). LME analysis revealed that there was a significant effect of the original plot L ($t = -3.81$, $P < 0.001$), but the effect of the transplanted plot L ($t = -0.19$, $P = 0.84$) and the interaction effect between the original and transplanted plots were not significant ($t = -0.23$, $P = 0.81$). This result indicated that the differences in the thermal requirement between populations might be determined by genetic regulation rather than plastic responses.

Discussion

Thermal requirement for flowering of *G. nipponica* was lower in the late-snowmelt population, resulting in a shorter preflowering period after snowmelt in comparison with the early-snowmelt populations. Reciprocal transplant experiments revealed that the GDD for FFD in original habitat was retained even after transplantation, while the actual preflowering period showed large plastic variation reflecting the environment of transplanted habitat. This result suggests that the genetic effect of flowering phenology may vary between local populations within a few hundred meters. A rapid flowering property is adapted to the late snowmelt populations, where successful seed maturation occurred in only 2/5 years owing to restricted season length. The present study is the first to detect the phenological regulation of flowering time to ensure reproductive success at a local scale. This finding indicates that the snowmelt gradient provides strong selective force on phenological traits of alpine plants, in addition to the occurrence of phenological isolation between populations.

Previous common-garden experiments demonstrated latitudinal differences in flowering schedule in *Arabidopsis thaliana* (L.) Heynh (Stinchcombe et al. 2004) and

Lythrum salicaria L. (Olsson and Ågren 2002). At high latitude and altitude, the short growth season may constrain the period available for seed maturation, and it should select for rapid flowering and fruiting (Olsson and Ågren 2002; Sandring et al. 2007). A similar environmental situation exists in the late-snowmelt habitat of the alpine ecosystem. On the other hand, early flowering at the early-snowmelt habitat increases the risk of frost damage. Therefore, contrasting selective pressures may act on the flowering trait between early- and late-snowmelt habitats. Flowering of *G. nipponica* usually occurred about 1 and 2 months after snowmelt in the late- and early-snowmelt sites, respectively. According to the classification of life-history strategies of arctic and alpine plants (Molau 1993), *G. nipponica* is a typical late-flowering species. This life-history trait also encourages the strong selection for rapid flowering in late-snowmelt populations.

Snowmelt time is a fundamental factor that controls the flowering pattern of arctic and alpine plants (Kudo 1991; Kudo and Suzuki 1999; Forrest et al. 2010; Wipf 2010). However, its impact on actual flowering time varies depending on the temperature conditions after snow disappearance. Snowmelt time determines the time of growth initiation, while subsequent ambient temperature affects the rate of floral development of alpine plants (Kudo and Suzuki 1999; Molau et al. 2005; Wipf et al. 2006; Wipf 2010). The GDD increases gradually early in the season owing to cool weather, whereas it increases rapidly in later in the season (Kudo and Suzuki 1999). Flowering occurrence was strongly correlated with snowmelt time in the late-snowmelt habitat more than that in the early-snowmelt habitat (Fig.1). This is because temperature limitation on growth rate is moderate in the middle of summer. Therefore, a simple comparison of preflowering periods between populations is insufficient to detect the intrinsic phenological traits of individual populations (Fig.2).

Kudo and Suzuki (1999) reported that three late-flowering species (*Vaccinium vitis-idaea* L., *P. multivitatum*, and *G. nipponica*) decreased the thermal requirement for flowering in late-snowmelt sites in the same area as this study. Although the possibility of phenological adaptation to complete seed production under restricted season length was speculated, it was not clear whether these variations reflect genetic difference among local populations or plastic responses to specific conditions in the previous study. Comparison of the GDD for flowering of *G. nipponica* using the reciprocal transplant experiment successfully detected the genetic variation between early- and late- snowmelt populations in this study. Because the daily mean soil surface temperature in 2006 was 12–14°C in July and 14–16°C in August, the difference in the GDD for flowering corresponded to a 4–7 d advancement of flowering time in the late-snowmelt population. In fact, this difference in the GDD seemed to contribute to the setting of mature seeds in the late-snowmelt population in 2006.

As an internal factor affecting flowering time, plant size or resource availability of individuals may be important as mentioned before. Several studies demonstrated a size-dependent variation in flowering phenology (Pettersson 1994; Ollerton and Lack 1998; Sandring et al. 2007). Total flower number within inflorescences significantly correlated with the timing of first flowering in *G. nipponica*. Plants with more floral buds showed earlier flower opening. Therefore, variation in plant size is a component causing within-population variation in flowering time in this species.

Even when significant variation in selective force exists between neighboring populations, some genetic isolation mechanism is necessary between the populations for local adaptation. For instance, Sambatti and Rice (2006) revealed that *Helianthus exilis* is composed of locally adapted populations that diverge in many ecological attributes between riparian and serpentine populations but remain tightly linked by gene flow. Thus, despite strong selective differences between habitats, extensive gene

flow probably reduces the chance for genetic differentiation to occur between populations. Analyses of landscape genetic structure in several alpine herbaceous species (Hirao and Kudo 2004, 2008; Yamagishi et al. 2005) revealed that the effect of phenological isolation on genetic structures varied among species. Hirao and Kudo (2004) detected a significant genetic differentiation among *G. nipponica* populations along the snowmelt gradient, suggesting the importance of pollen flow dynamics via pollinators for the formation of genetic structure. Because seeds of this species are dispersed by gravity around the mother plants and seeds do not have any device for secondary dispersal, the distance of gene flow via seed dispersal may be small. Therefore, the isolation of flowering season caused by the difference in snowmelt time may maintain genetic variation between neighboring populations in this species.

Because we used plants grown in their original habitat in the transplant experiment, we cannot completely rule out the possibility that developmental plasticity may lead to a specific patterns of bud formation in the transplanted habitat. However, transplanted plants showed consistent phonological patterns over two seasons. This indicates the importance of genetic regulation on the phenological variation between local habitats, although the transplanted experiment or the common garden experiment based on progeny plants is most preferable to discriminate the environmental effect and genetic effect on phenology. Clarification of the genetic background controlling flowering phenology and the maintenance of genetic variations among neighboring populations is necessary to elucidate the mechanisms for local adaptation in this species.

Acknowledgements

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Figure captions

Fig. 1. Reproductive phenology of *Gentiana nipponica* in each plot from 2005 to 2009.

Dots, solid columns, and open columns indicate snowmelt time, flowering period,

and fruiting period, respectively. Crosses mean there was no flowering or fruiting

within the plot.

Fig. 2. Reaction norms of (a) first flowering day (FFD) and (b) effective cumulative

temperatures from snowmelt to FFD of transplanted *Gentiana nipponica* between

early- and late-snowmelt plots in 2006. Box plot represents the 75th, 50th and 25th

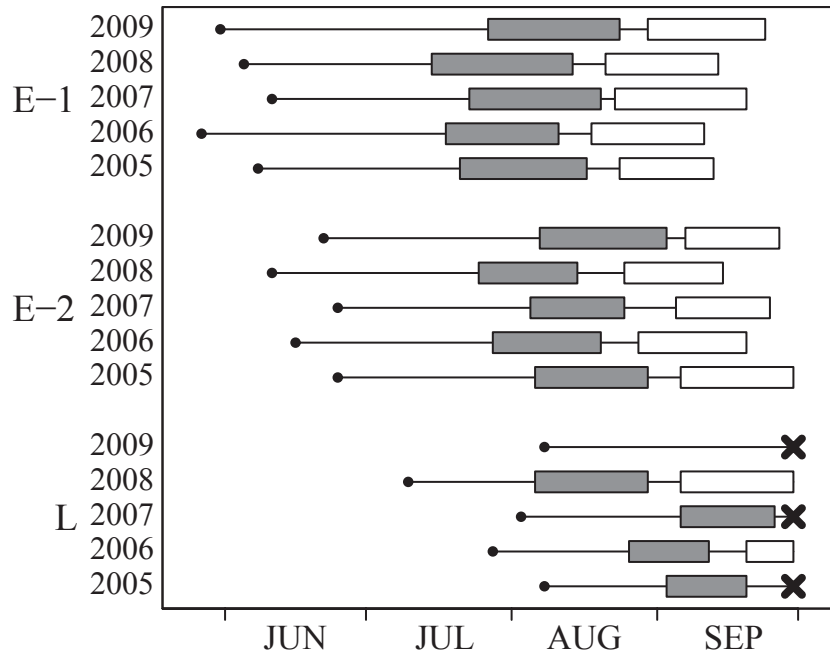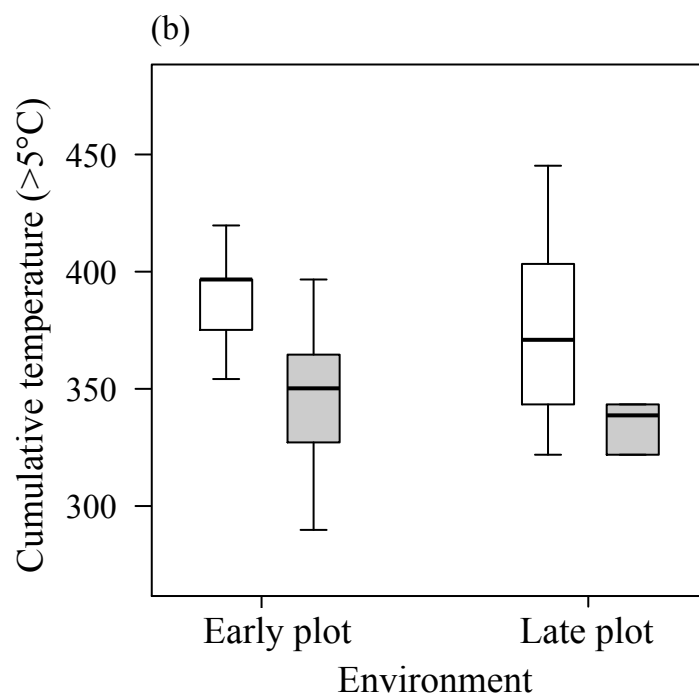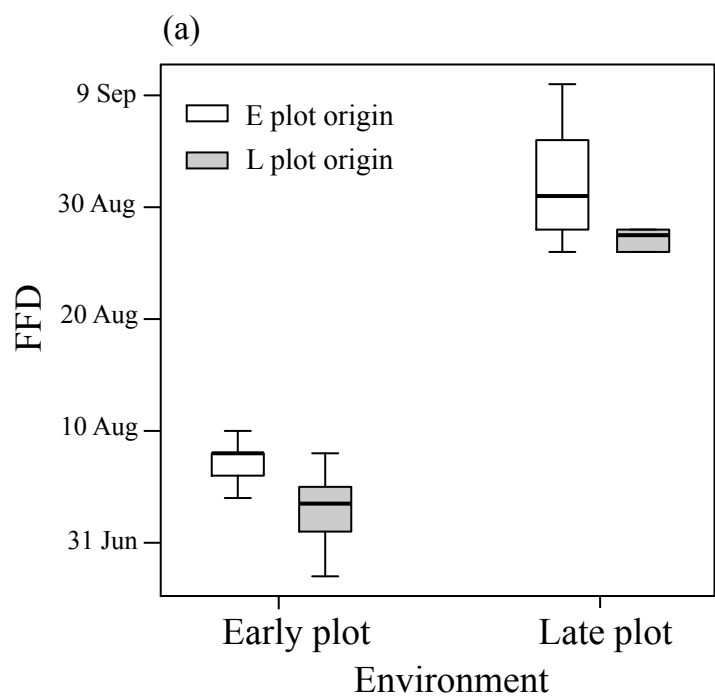percentiles. The lines joined to the box extend to 1.5 × the interquartile range.

Figure 1

Figure 2   Yuka Kawai and Gaku Kudo

(a)

(b)

Tables

Table 1. Description of flowering phenology in the three study plots.

	Plot			
	E–1		E–2	L
Year	2005	2006	2005	2006
Snowmelt date	8 June	27 May	25 June	28 July
Sample size	46	50	31	100
First flowering day	24 July	18 July	6 August	26 August
Preflowering period (d)	51.6±6.1	60.9±4.5	45.5±1.7	29.2±3.2
Growing degree days	377±56	376±38	392±21	323±34

Note: E, early-snowmelt plot; L, late-snowmelt plot; sample size, the number of reproductive shoots used for comparisons; preflowering period, the number of days from snowmelt to first flowering; growing degree days, a threshold value of 5°C. Mean ± SD are given for preflowering period and growing degree days values.

Table 2. Description of flowering phenology in the reciprocal transplant experiment between 2006 and 2007.

	Treatment			
	E to E	L to E	E to L	L to L
Original plot	E–2	L	E-2	L
Transplanted plot	E–2		L	
2006				
Snowmelt date	18 June		28 July	
Sample size	9 (10)	14 (24)	8 (14)	10 (18)
Preflowering period (d)	51.7±2.6	47.9±2.6	35.1±4.7	30.1±1.0
Growing degree days	384±27	345±30	376±37	333±11
2007				
Snowmelt date	25 June		3 August	
Sample size	2 (3)	No flower	4 (5)	4 (8)
Preflowering period (d)	53.0±1.7	—	47.0±3.0	39.8±1.2
Growing degree days	464±17	—	348±17	305±8

Note: E, early-snowmelt plot; L, late-snowmelt plot; sample size, the number of clumps (the total number of reproductive shoots) used for experiments; preflowering period, the number of days from snowmelt to first flowering; growing degree days, a threshold value of 5°C. Mean ± SD are given for preflowering period and growing degree days values.