Carbon allocation strategies for reproduction and growth in spring ephemeral plants

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Contents

Summary

Spring ephemerals constitute part of the herb layer in temperate deciduous forests and they are characterized by a relatively short growth period from snowmelt to canopy development of overstory vegetation in early summer. These plants are faced with declining light availability with leaf emergence of canopy trees. They generally have overlapping periods of growth, reproduction, and concurrently refilling the storage organs with carbohydrates. Spring ephemerals commonly have high reproductive output even under short growth period, but the carbon allocation strategies and mechanisms responsible for active reproductive output have been studied only for restricted species. In addition, the effects of decreasing light intensity and changing environmental conditions on both vegetative and reproductive functions are poorly understood. The purpose of this study is to clarify the mechanisms of reproductive compensation and carbon allocation under fluctuating light and temperature environments in a typical spring ephemeral species, *Gagea lutea* (Liliaceae).

In Chapter 1, the mechanism to mitigate the cost of reproduction was clarified by monitoring leaf and bract performance, bulb growth, and seed production under natural condition. Leaf growth, foliar and non-foliar (bract) photosynthetic activities, and total carbon assimilation were compared among reproductive-intact, floral-bud removal, and vegetative plants. Translocation of current photosynthetic products to individual organs was quantified by a $^{13}CO_2$-trace experiment. Bulb growth was compared between hand-pollinated and floral-bud removal treatments and seed set was compared between intact, leaf-clipping and bract-clipping treatments. Fruit-forming plants retained leaves longer than vegetative and floral-bud removal plants, but the assimilative contribution of extended leaf longevity was negligible. Carbon

supply by bract photosynthesis was large enough for fruit development. Leaf photosynthetic products were largely transported to bulbs. The leaf-clipping had no effect on seed production, while the bract-clipping significantly reduced the seed production. Therefore, current photosynthesis of leafy bracts was a major carbon source for fruit development. This self-compensative mechanism of reproductive structure enables the continuous reproductive activity in this species.

In Chapter 2, the effects of shading by early canopy closure on reproductive output and vegetative growth were clarified. With a bract-removal treatment (source reduction) and a floral-bud removal treatment (sink reduction) under canopy and open conditions, the effects of sink-source balance on seed production and bulb growth were investigated. Leaf carbon fixations did not differ between the forest and open sites and among treatments. Bract carbon fixations were also similar between sites but tended to decrease when floral buds were removed. Seed production was higher under open condition but decreased by the bract-removal treatment under both light conditions. Although bulb growth was independent of light conditions and the bract-removal treatment, it was increased greatly by the bud-removal treatment. Therefore, leaves and bracts acted as specialized source organs for vegetative and reproductive functions, respectively, but photosynthetic products by bracts were flexibly used for bulb growth when plants failed to set fruits. Extension of bright period was advantageous for seed production (i.e., source limited) but not for vegetative growth (i.e., sink limited) in this species.

In Chapter 3, the effects of temperature on reproduction and vegetative growth were evaluated to predict the fate of spring ephemerals under warming climate. Although previous studies reported better performance in some spring ephemerals grown under cool conditions, most of these studies were conducted only for non-

reproducing plants. In the present study, therefore, leaf physiological activities, bulb growth, and seed production were compared among reproductive plants grown at the forest, open, and greenhouse (GH) plots. Solar radiation, soil temperature, and air temperature showed higher values in the GH leading to earlier snowmelt and growth initiation. Leaf and bract photosynthetic activities decreased rapidly at fruiting stage but dark respiration remained at high level in the GH, resulting in larger carbon consumption during fruit development under warm conditions. Although initial bulb size was not different among plots, final bulb size was largest at the forest plot and smallest at the GH plot. Reduced seed set was recorded only at the GH plot. Therefore, decreasing population dynamics of spring ephemerals was predicted under warming climate.

This research provides new explanation for the mechanisms responsible for high reproductive output in spring ephemerals. Carbon assimilation under continuous bright condition may be beneficial for reproduction only when early canopy closure restricts the carbon assimilation of reproductive function, but this is not the case for vegetative growth. This means that too early canopy closure may influence the seed production of spring ephemerals. Warmer temperature, on the other hand, could reduce both vegetative growth and seed production, indicating that spring ephemerals may be the first species to be negatively affected by global warming.

General Introduction

Temperate deciduous forests in the Northern hemisphere commonly encompass multiple layers of vegetation. Tallest trees make up the forest canopy, which can be 30 m or more above the ground. Beneath the canopy layer, small and young trees compose the sub-canopy layer. Below the tree layer, understory layer exist that is composed of shrub, herbaceous, fern and moss species. Light availability in the understory varies greatly as the season progresses. On the forest floor, high light intensities persist in early spring prior to the leafing out of canopy trees, but with canopy closure, decreased light condition sets in and lasts till autumn when deciduous broad-leaves are shed.

In northern deciduous forests, spring ephemerals are a common element of the herb layer. Most of these species are clonal and perennial (Whigham 2004), and are characterized by a relatively short lifespan between snowmelt and canopy closure in early summer (Lapointe 2001). During this period, these plants are faced with declining light availability due to leaf emergence of canopy trees. In addition, they generally have overlapping periods of growth, reproduction and concurrently refilling the storage organs within the short growth period. Aboveground shoots senesce around the time of canopy closure and belowground parts become dormant until next growth phase begins. Therefore, reasonable allocation of photosynthetic products during the growth period is important for plants to perform efficiently.

Since reproductive output is apparently an important component of fitness, various trade-offs exist between life-history variables in terms of reproduction (Reznick 1985; Obeso 2002). The concept of reproductive cost assumes that the total cost of reproduction in a given year can transform into somatic and/or demographic

costs the following year (Jönsson 2000). Some studies have documented the evidence of reproductive cost (Obeso 1993; Primack and Stacy 1998; Gehring and Delph 2006; Newell 1991; Nicotra 1999), while others have found little evidence (Dudash and Fenster 1997; Ramsey 1997). These contradictory results can be explained by the fact that reproducing individuals may increase their resource intake and develop some compensatory mechanisms for fruit and seed production. In spring ephemerals, minimum number of leaves, short growth period and low light conditions may restrict their reproductive output. Nevertheless, previous studies reported that they normally have high fruit set (Kudo *et al*. 2008; Bernett *et al*. 2009). Therefore, they should have specific means of ensuring the high reproductive output under such reduced growth period with limited number of leaves. Even though it is essential to pin point the extent of trade-offs between current reproduction and future performance to understand life-history variations, less attention has been given to the detailed evaluation of carbon assimilation and compensatory mechanisms in spring ephemerals.

Spring ephemerals are known for a characteristic high photosynthetic rate in early spring due to high light intensities incident on the forest floor prior to canopy closure (Taylor and Pearcy 1976; Lapointe 2001). Assimilated carbon during this active and short period may be channeled simultaneously to fulfilling both vegetative and reproductive performances thereby creating a trade-off relationship in carbon allocation between reproduction and growth. Periodicity in resource allocation during the growing season has been documented in several herbaceous species (Lapointe 1998; Garcia and Ehrlen 2002; Horibata *et al*. 2007; Ida and Kudo 2008; Kudo and Ida 2010; Kleijn *et al*. 2005). Species with a brief photosynthetic period and/or low resource availability like spring ephemerals often depend on storage for reproduction

(Wardlaw 1990; Kudo and Ida 2010). Leaf senescence in spring ephemeral species corresponds with the timing of forest leaf display. Hence, overall carbon gain during the growth period may be limited, thereby increasing the trade-offs between vegetative growth and reproduction. In addition, if present resource demand by reproductive sink surpasses current gain by assimilative source, reproductive function may compete with vegetative function for limited resource use. However, if sink and source organ can operate independently, reproduction and vegetative growth may not compete for resource use. Therefore, explicit investigation of source and sink activity is necessary to understand the extent of trade-offs between reproduction and vegetative functions.

Despite the rapid decline of light level in late season, they commonly have high fruit set (Kudo *et al*. 2008) especially at the forest edge (Nishikawa 2009). It is interesting that a high reproductive output was reported at the forest edge and that makes it vital to clarify in detail the effect of low light condition under the canopy on the overall plants fitness but little is known about this. Furthermore, establishing the broad sink-source responses to the decreasing light condition under the forest is important in the study of spring ephemerals' life-history and resource utilization.

As the name "spring ephemeral" implies, these species grow early in spring when temperature is low and could be limiting their growth. At low temperatures, growth rate and final size are usually reduced even for plants adapted to cool environments (Körner and Larcher 1988). Temperature has been a major factor affecting the growth of plants (Grace 1988). Enzyme activity and cell division decrease at cool temperature (Fitter and Hay 1987; Tardieu *et al*. 2000), but higher temperatures enhance enzyme activity and photosynthesis. Nevertheless, previous studies have shown that spring ephemerals grow better at cool temperatures (Wurr *et*

al. 1998; Lapointe and Lerat 2006; Badri *et al*. 2007; Gandin *et al*. 2011). High biomass buildups under cool temperatures reflect the adaptation of spring ephemerals to the cold spring temperatures but they might be at risk under warm springs as predicted under future climate change. Initiation of growth and flowering in spring ephemerals depend mostly on snowmelt timing and air temperature hence, their life cycle is most sensitive to spring thermal conditions (Diekman 1995).

If spring begins earlier due to global climate change, it may increase the length of active photosynthetic period, provided the end of season remain unchanged. This might translate into a positive carbon gain for spring ephemerals. Since leafing out of trees is occurring earlier also due to warmer spring temperatures (Menzel 2000), however, shading effect could restrict the photosynthetic rate, carbon assimilation and fruit production to some extent. It is necessary therefore to investigate the detail response of plants to warmer spring temperatures. Previous researches have worked only on non-flowering individuals, however, the phenology of reproduction is an important life-history trait that relied mostly on environmental cues and cannot be neglected when predicting plants response to climate change.

By monitoring leaf size dynamics, bulb growth, photosynthesis, carbon fixation, plant growth and reproduction, this project investigates the strategy of resource allocation and its sensitivity to growth conditions in a spring ephemeral plant *Gagea lutea.* In the first chapter, I focused on the relationship between current reproductive performance and vegetative growth in terms of reproductive cost. In the second chapter, the extent of trade-offs and integration between reproduction and growth under different light conditions were clarified in order to reveal the effect of shading by early canopy closure on the sink-source activities. The third chapter elucidated the probable response of reproduction and growth to the predicted global climate change.

Lifetime fitness depends mostly on the timing of life-history traits hence; early snowmelt and warmer spring attributable to climate change should have some significant consequences for spring ephemerals. I quantified such consequences especially on the storage organ and seed production. Based on these, I discussed the efficient resource use in this species in relation to other understory plants.

Study Species and Site

Gagea lutea Ker-Gawl. (Liliaceae) is a polycarpic perennial herbaceous species in the northern temperate forests. It is distributed across China and extends from Europe to Japanese Islands. This species has a typical spring ephemeral lifecycle; blooming starts immediately after snowmelt concurrently with leaf expansion (in mid- to late April) and fruits mature about two weeks after anthesis. It produces 1-10 flowers and 24-39 ovules per plant on average (Nishikawa 1998, 1999). Early blooming flowers tend to be bigger than late blooming ones. Aboveground parts usually die at the time of seed dispersal by canopy closure in late May. Thus, the short period between snowmelt and canopy closure is when this species accumulate resources in the underground perennial organ.

Vegetative (non-reproductive) individuals produce only one leaf, while one basal leaf and a pair of long and short leaf-like bracts on the scape are produced in reproductive plants (Fig. I). Each reproductive shoot bears multiple flowers (3.4 ± 0.2 S.E., n = 40) on a single scape that bloom sequentially (Nishikawa 1998). The underground part (bulb) is a storage organ, which is the only tissue existing over years. *G. lutea* is pollinated by insects and cross-pollination is more effective for seed production.

The first experiment was conducted in a secondary deciduous forest within the campus of Hokkaido University, Sapporo, northern Japan (43° 04' 57" N, 141° 20' 22" E). Bulbs used for subsequent experiments were collected from the same forest. This forest is usually covered with snow from early December to early April. Major canopy trees in this fragmented forest include *Ulmus davidiana* var.*japonica*, *Cercidiphyllum japonica*, *Betula platyphylla* var. *japonica* and *Populus maximowiczii*

and the forest floor is always covered with *G. lutea* together with other spring ephemerals in the spring.

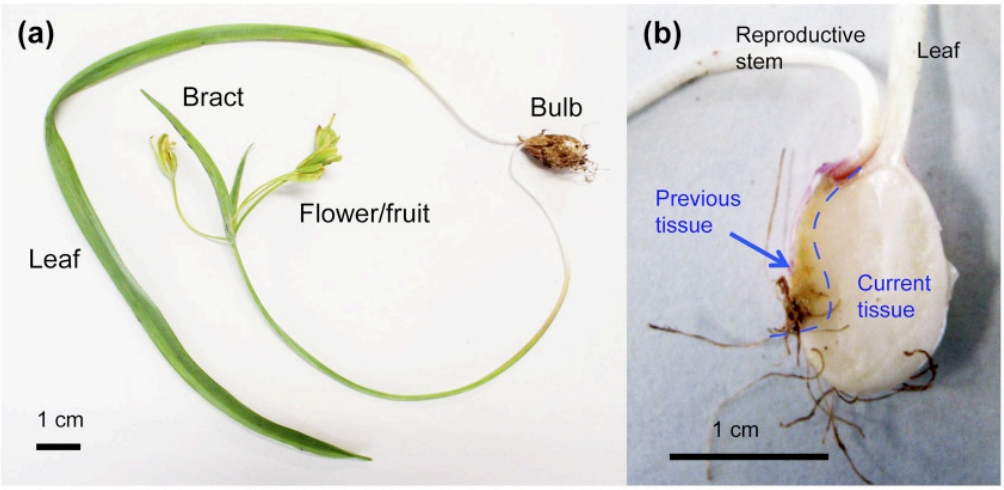

Figure I. Photograph of *Gagea lutea*. (a) Whole plant structure at early fruiting stage. (b) Section of a bulb at early fruiting stage. Reproductive stem is connected to previous tissue that has been exhausted, while leaf is connected to developing current tissue.

Chapter 1.

Photosynthetic Compensation by the Reproductive Structures

1.1. Introduction

Reproduction requires substantial amount of resources and this makes the plants manage the process of reproduction effectively (Banuelos and Obeso 2004). Such the gross material costs of reproduction often result in a decrease in allocation to non-reproductive functions (Wardlaw 1990), and occur at the expense of somatic investment that may cause a decline in survival and/or future reproduction, i.e., demographic cost of reproduction (Reznick 1985). Thus, the concept of cost of reproduction assumes that the gross cost of annual reproduction can translate into demographic cost (Obeso 2002). As mentioned in General Introduction, the lack of reproductive cost in several studies (Dudash and Fenster 1997; Ramsey 1997) may be explained by at least three compensative mechanisms for resource provision to seed production. Firstly, temporal variation in sink-source pathway may prevent a simple trade-off between resource allocation to current seed production and storage for future performance. Early-blooming forest herbs, for instance, commonly construct the aboveground structure using resources previously stored in the underground parts **(**Muller 1978; Routhier and Lapointe 2002), but this function changes afterwards to sink for future activities (Geber *et al*. 1997; Ida and Kudo 2008). Because the translocation of annual photosynthetic products may vary temporally (Ida and Kudo 2008), the relative contributions of current photosynthesis and stored resources to reproduction should also vary within a reproductive period (Ida and Kudo 2008; Kudo and Ida 2010).

Secondly, the resource demand of reproductive sink may elevate the assimilative capacity of source function (Watson and Casper 1984; Wardlaw 1990). If a carbon supply from photosynthetic organ is controlled by the demand of sink organs (Wardlaw 1990), a large part of carbohydrates may be supplied by the increased photosynthesis of adjacent leaves (Gifford and Evans 1981) or overall increase in photosynthetic ability (Wardlaw 1990; Lehtilä and Syrjänen 1995) although such up-regulation of photosynthesis may not be universal (Watson and Casper 1984). Furthermore, the extension of leaf lifespan during the reproductive period may contribute to assimilative capacity. For instance, larger reproductive cost in female individuals of dioecious trees than male individuals (Lloyd and Webb 1977; Obeso 2002) is compensated by longer leaf life-span and/or larger leaf size (Jonasson *et al*. 1997; Tozawa *et al*. 2009) in addition to the elevation of photosynthetic capacity.

Finally, the reproductive structures themselves may also boost resource uptake during reproduction. Significance of photosynthetic carbon gain by non-foliar organs, such as greenish flowers, developing fruits, green petals and stem tissues, has been reported (Blanke and Lenz 1989; Salopek-Sondi *et al.* 2000; Aschan and Pfanz 2003; Herrera 2005). These photosynthetic parts associated with inflorescences can reduce the gross cost of reproduction to some extent (Marcelis and Hofman-Eijer 1995; Antlfinger and Wendel 1997). However, because the net photosynthesis by reproductive structures may be canceled by their high respiration rate (Watson and Casper 1984; Obeso 2002), careful considerations are required to clarify the extent of compensative capacity of non-foliar structures.

Spring ephemerals inhabiting deciduous forests often have small number of leaves and very short leaf longevity. They are faced with a declining photosynthetic carbon gain because light availability consistently decreases from spring to early

summer due to leaf expansion of canopy trees. Nevertheless, previous studies demonstrated that spring ephemerals commonly have high fruit production (Kudo *et al*. 2008; Bernett *et al*. 2009). Thus, they should have some mechanisms to ensure the high reproductive output under restricted growth period with limited number of leaves. This is partly related to high photosynthetic rates under high irradiance before canopy closure (Rothstein and Zak 2001; Kudo *et al*. 2008) and/or use of storage resource instead of current assimilation (Horibata *et al*. 2007; Kudo and Ida 2010). To clarify the reproductive strategies of carbon assimilation in spring ephemerals, however, intensive assessment of compensative mechanisms is required.

This chapter therefore is aimed at answering the following questions: (a) Does the resource provision to seed production translate into a trade-off between current reproduction and storage for future growth? (b) Do the plants elevate foliar photosynthetic capacity, such as the increments of size, longevity and/or photosynthetic rate, to satisfy the carbon demand for seed production? (c) Does the non-foliar (young fruits and bracts) photosynthesis substantially contribute to seed production? With these questions, I can evaluate the kind of compensative mechanisms for seed production that exists in *Gagea lutea* and to what extent the annual cost of seed production is assured. Although a previous study on *G. lutea* suggests the importance of current photosynthetic products for seed production (Nishikawa 2009), the responses of seed production to defoliation treatments were inconsistent between years. Nevertheless, since reproductive individuals of this species always have a pair of leafy bracts on a flowering stem, it is anticipated that bracts may indicate an important compensatory strategy to foster carbon gain during reproduction. Based on carbon fixation and dry weight analysis, I aimed to clarify the

demographic cost of reproduction and compensative strategies, and to quantify the resource for seed production.

1.2. Methods

Photosynthetic activity

To assess the photosynthetic capacity of basal leaves, we selected 3-4 plants per reproductive status at four growth stages: pre-flowering stage on 16 April, flowering stage on 5 May, early-fruiting stage on 18 May, and late-fruiting stage on 30 May (only for reproductive-intact plants) in 2010. Furthermore, basal-leaf photosynthetic capacity of vegetative individuals (at 16 April, 5 May, and 18 May) and bud-removal individuals (at 5 and 18 May; mentioned later) was measured as same as reproductive intact plants. In addition, fruit photosynthesis of same reproductive-intact plants was measured at early- and late-fruiting stages in 2010. To assess the photosynthetic capacity of bracts, we selected three reproductive-intact plants at each of five growth stages: floral-bud stage on 14 April, early-flowering stage on 28 April, late-flowering stage on 11 May, early-fruiting stage on 20 May, and late-fruiting stage on 31 May in 2011. We measured light responses of photosynthetic rates per unit area using LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NB, USA). Ten light conditions (2000, 1500, 1000, 800, 500, 300, 100, 50, 10, and 0 μmol m^{-2} s^{-1}) of PAR were provided using a red-blue LED light source at constant leaf temperature (15°C). Ambient CO_2 concentration and the humidity of incoming air during the measurement were maintained at 380 μl l^{-1} and 1.1 vapor pressure deficit (VPD, kPa), respectively. Net photosynthetic rate per area (P_{area}) can be described as a non-rectangular hyperbola of photon irradiance (I, μmol m^{-2} s^{-1}) as follows:

$$P_{area} = \frac{\alpha I + P_{\max} - \sqrt{(\alpha I + P_{\max})^2 - 4\alpha I \theta P_{\max}}}{2\theta} - R_d \quad (1)$$

where P_{max} is the light-saturated photosynthetic rate per unit area (μmol m^{-2} s^{-1}), α is the initial slope (μmol m^{-2} s^{-1}), θ is the degree of curvature, and R_d is the dark respiration rate (μmol m^{-2} s^{-1}; Marshall and Biscoe 1980). Data obtained for individual plants were fitted to this equation by non-linear least-squares estimates of the parameters. Mean values of individual parameters obtained in each period were used as representative of photosynthetic capacity. Because the width of basal leaves and bracts was smaller than the chamber size (2 cm × 3 cm), we corrected photosynthetic parameters by the replacement of chamber area by actual leaf area that was included in the chamber.

Carbon fixation capacity

To assess the effects of reproductive activity on assimilation capacity, we compared total carbon fixations by basal leaves and bracts among different reproductive status (reproductive-intact, bud-removal, and vegetative plants). Soon after shoot emergence (8 April 2010), 40 plants with floral buds and 20 plants without floral buds but having similar-sized leaves to the former (vegetative plants) were arbitrarily selected and marked with numbered tags. After recording the number of floral buds, all buds were removed from the 20 of reproductive plants (bud-removal plants). All buds of the remaining 20 plants (reproductive-intact plants) were retained and supplemental pollination was conducted. Pollen donors for a supplemental pollination were selected from multiple reproductive plants >5 m apart from the recipient plants. We therefore

compared the assimilation capacity among reproductive status. First, to assess the seasonal changes in photosynthetic area, the leaf and bract sizes (length, L and width, W) of all plants were measured with a digital calliper at 5-day intervals except on rainy days throughout the growth period. This measurement involved only the green area that was photosynthetically active. Basal leaf and bract area (A) was estimated as $A = 0.83 \times L \times W$ ($r^2 = 0.968$, $n = 5$). Also, surface area of harvested fruits was measured with the image analysis software (Image J version 1.34; National Institutes of Health, Bethesda, MD, USA). Furthermore, leaf senescence pattern of each plant was recorded to quantify the leaf longevity.

The total carbon fixation by basal leaves, bracts, and fruits throughout the season from the data of photosynthetic area, photosynthetic parameters, and light availability were determined. The 1-min PAR data measured at the study site was converted to hourly mean values before the estimation. Net photosynthetic rates per unit area per hour (μmol m^{-2} s^{-1}) were estimated based on the light availability and photosynthetic parameters obtained at each growth stage (four, five, and two stages for leaves, bracts, and fruits, respectively) explained above. Hence, daily CO_2 fixation rate (mmol m^{-2} day^{-1}) was expressed as the sum of hourly photosynthetic rates, calculated using the equation (1) with hourly mean PAR and photosynthetic parameters of each reproductive status in each period. In this calculation, daily transitions of photosynthetic area were estimated as a proportional increase or decrease between days of measurements. Then, by summing the daily CO_2 fixation rate multiplied by photosynthetic area during the growth period (average leaf longevity of each reproductive status), total carbon fixation (mg C) by basal leaves, bracts, and fruits were obtained. Carbon concentration in plant tissue of *G. lutea* was 45% of dry mass and the conversion efficiency of energy required to transform glucose or sucrose into

the different cell components is supposed to be about 70% in general plant tissues (e.g. Poorter and Villar 1997). Thus, total biomass gain caused by the photosynthetic carbon fixation (C-fixed) was calculated as C-fixed × 1/0.45 × 0.7.

Growth pattern

To compare the seasonal growth patterns between vegetative and reproductive plants, the dry weight of each organ was measured by harvesting 15 plants per status at each of four stages (bud, flowering, early fruiting, middle fruiting) during the growth season of 2012. Individual plants sampled were then taken to the laboratory and separated into two (leaf and bulb) for vegetative and four organs (leaf, bract, flowering stem, bulb including roots) for reproductive plants. Flowering stem composed of stem and flower during flowering but stem and fruits during fruiting season. Roots were added as part of the bulb because they had negligible masses. Leaf length and width were measured immediately after the separation before leaves folded up with a digital caliper. Leaf area was later calculated non-destructively following the same equation as stated above. Afterwards, each sample was oven-dried at 70°C for 72 h and weighed.

$^{13}CO_2$ labeling

To clarify the allocation pattern of current photosynthetic carbon fixed by basal leaves to individual organs, ^{13}C-trace experiment was performed at flowering (1 May, n = 9) and late fruiting (28 May, n = 4) periods by supplying $^{13}CO_2$ to leaves for two days in 2010. ^{13}C levels for five individuals without $^{13}CO_2$ exposure were also measured as a control in each period. For this experiment, leaves were enclosed in a 40 × 30-cm sealed nylon bag in which a cylinder containing 30 mL of lactic acid and two tubes

containing 150 mg of 99.9% $Ba^{13}CO_2$ (Isotec Inc., Miamisburg, OH, USA) were present. The $^{13}CO_2$ labeling was carried out when there was sufficient sunlight to cause net CO_2 uptake, on two successive sunny days. Injections of $^{13}CO_2$ were made in early morning on the first and second days. Barium carbonate was added to lactic acid, releasing $^{13}CO_2$ into the bag. Plants were harvested two days after the labeling period. After harvesting, individual plants were separated into six organs (flower or fruit, stem, leaf, bract, bulb and root) and oven-dried at 70°C for 72 h. After weighing each organ, they were ground in a mortar. The combined system of an elemental analyzer (Flush EA; Thermo Fisher Scientific, Bremen, Germany) and an isotope ratio mass spectrometer (Delta V Plus; Thermo Fisher Scientific) was used to measure $\delta\ ^{13}C$ (see Ida and Kudo 2008 for details). Because dry mass and ^{13}C contents were very small in roots, we excluded root data from the analysis. The distribution of excess ^{13}C via photosynthesis was expressed with the proportion of excess ^{13}C in each organ against total absorbed ^{13}C per plant.

Bulb growth between years

Bulb size between hand-pollination (maximum fruit set) and floral-bud removal (no flower and fruit production) treatments were compared to assess the effects of reproduction on bulb growth. Bulbs of 34 plants were unearthed on 20 November 2009 in the forest floor, then the length (L) and width (W) of individual bulbs were measured using a digital calliper (initial bulb size). Because the shape of bulbs was non-uniform (ellipsoid), the mean of the two broader widths was taken as the width, and the bulb volume was expressed as $4\pi \times W^2 \times L / 3$. After the measurement, they were planted in pots with numbered tags for identification. The pots were put in holes

under the forest so that bulbs could continue to grow at the same depth as they normally grow and under natural climate conditions.

Occasional wetting was performed to prevent evaporation. In early spring of 2010, plants having floral buds (n = 28) were divided into two groups. All flowers of the first group (n = 15) were hand-pollinated to set maximum fruits, while floral buds of other group (n = 13) were eliminated to remove the resource provisioning to flowering and fruiting. Hand-pollination was performed by brushing the dehisced anthers from donors >5 m away across the clear stigma of the flowers using forceps until the surface of stigmas was completely covered with pollen. The floral-bud number was recorded before the bud removal, and the sizes of basal-leaf and bracts of every plant were measured during the fruiting season in mid-May (at maximum size) using a digital calliper. On 14 October 2010, individual bulb sizes were measured again (final bulb size). Then, final bulb volume was analysed with the consideration of reproductive activity and initial bulb size.

Annual reproductive output

To investigate the carbon source for seed production, 60 well-pollinated plants with three developing fruits were selected. The selected plants, identified with number tags, were divided into three groups of 20 individuals each (intact control, bract-clipping, and leaf-clipping). Clipping was performed just after the initiation of fruit development. On 7 June 2010, infructescences were harvested from all plants before the opening of capsules. Fruit, undeveloped ovule and mature seed numbers were counted. Then, effects of clipping on fruit-set rate (fruit/flower ratio) and seed-set rate per plant (seed/ovule ratio) were assessed.

Statistical analyses

An open source system, *R* version 2.12.1 (R Development Core Team 2011) was used for all statistical analyses. I used one-way analysis of variance (ANOVA) to compare leaf size and leaf carbon fixation among three treatments, respectively. Turkey's HSD test was used for post-hoc multiple comparisons. Relationships between flower number and leaf size, and between carbon fixation and leaf size were determined using Pearson's correlation coefficients. Leaf size and leaf carbon fixation were log-transformed before analysis to improve normality. Each of bract size, bract carbon fixation, and bulb size was compared between the reproductive-intact and bud-removal treatments using Student's *t* test without any data transformation. Relationships between flower number and bract size, and between carbon fixation and bract size were determined using Pearson's correlation coefficients. Before conducting ANOVA or *t* test, I confirmed the normality of data distribution and homogeneity of variances by Shapiro-Wilk test and Bartlett test, respectively. Leaf survival rate was compared among three treatments using the Cox proportional hazards regression model. To compare the photosynthetic activity among three treatments, I applied a generalized linear model (GLM) postulated a gamma error distribution with log-link function to P_{max} values of basal leaves in which treatment and growth stage (pre-flowering, flowering, early fruiting) were set as explanatory variables. I compared organ development among growth stages by Holm's multiple comparison since the data is neither normal nor had equal dispersion. Fruit-set (fruit/flower ratio) and seed-set (seed/ovule ratio) were compared among clipping treatments by GLM postulated a binomial error distribution with logit-link function in which intact was set as an intercept, and leaf and bract clipping as explanatory variables.

1.3. Results

Growth pattern

Allocation patterns differed between reproductive and vegetative plants and individual organs varied within the season. In the vegetative plants, leaf area and leaf mass increased from first to second sampling, these were maintained until third sampling but later increased during the fourth harvest (Fig.1-2a). Bulb volume and mass were similar between first and second sampling indicating the dependence of shoot production on previous storage. As the season progressed, bulb volume and mass increased significantly showing the renewal of the perennial organ. Mean leaf and bulb masses follow the same trend (Fig.1-1b). Overall, total biomass increased consistently across the season (Fig.1-2a). In the reproductive plants however, both leaf and bract area increased from budding to flowering and remained unchanged till middle fruiting period (Fig.1-2b). Leaf and bract mass follow the same trend but decreased at middle fruiting stage indicating the initiation of senescence. Flowering stem mass did not increased significantly from bud to flowering stage but increased during early fruiting and maintained until middle fruiting stage (Fig.1-2b). This result signifies the development of floral organ from flower to fruit.

On the contrary, bulb volume and mass exhibited a decrease from bud to flowering stage explaining the total dependence of shoot and flower production on the previously stored resources. Afterwards, bulb volume increased during fruiting and bulb mass even revealed an increment from early to middle fruiting. Mean bulb, leaf, bract and flower stem masses showed similar trends (Fig.1-1a). Overall, total biomass was similar between bud and flowering stages but increased significantly as the season progressed (Fig.1-2b).

Leaf and bract growth

Basal leaves grew continuously until early-fruiting stage (mid-May), then the green area decreased with progress in leaf senescence from the tip, and died back completely by the end of May (Fig.1-3a). Vegetative plants had significantly smaller leaves than reproductive-intact and bud-removal plants, while there was no significant difference between intact and bud-removal treatments (Table 1-1). There was a positive correlation between maximum leaf size and potential flower number ($r = 0.68$, $P < 0001$), indicating size-dependent reproductive investment.

Bracts were less than 20% of basal leaves at the maximum size, and bract size was rather stable during the growth period than basal leaf size (Fig.1-3a). Similar to basal leaves, bract size did not differ between intact and bud-removal treatments (Table 1-1), and there was a positive correlation between maximum bract size and potential flower number ($r = 0.75$, $P < 0001$).

Basal leaves of both the bud-removal and vegetative plants senesced significantly earlier than leaves of reproductive-intact plants (Fig.1-3b). This indicates that reproductive-intact plants had longer leaf life-span (54.7 ± 1.1 S.E. days) than bud-removal (49.6 ± 0.8 days) and vegetative plants (49.3 ± 0.8 days).

Photosynthetic activity

The light-saturated photosynthetic rate per unit area (P_{max}) was high at the pre-flowering and flowering stage then, decreased rapidly during the fruiting period for all treatments (Fig.1-4a). Result of GLM indicates that there was no significant difference in P_{max} among treatments but P_{max} significantly differed among growth stages (Table 1-2). Only the reproductive-intact plants survived and photosynthesized until late-fruiting period although the activity was very low (P_{max} was only 7% of

flowering period). Dark respiration rate (R_d) was relatively stable throughout the growth period ranging from 0.9 to 1.8 μmol m^{-2} s^{-1}.

Similar to basal leaves, bracts showed high P_{max} at the pre-flowering and flowering stages, then P_{max} constantly decreased during the fruiting period (Fig.1-4b). Developing fruits showed relatively high P_{max} (6.4 μmol m^{-2} s^{-1}) comparable to bract P_{max} (6.2 μmol m^{-2} s^{-1}), but their R_d was much higher (2.7 μmol m^{-2} s^{-1}) than bract R_d (0.4 μmol m^{-2} s^{-1}) at the early fruiting stage. Appendix 1-1 and 1-2 shows the details of photosynthetic parameters.

Carbon fixation

Seasonal transitions of daily CO_2 fixations of individual organs reflected the fluctuation of daily maximum PAR (Fig.1-5). Basal leaves and bracts showed similar carbon fixation per unit area, remaining high in April but decreasing rapidly after mid-May with progress of canopy closure. In contrast, photosynthetic CO_2 fixation by fruits was almost zero or often negative throughout the fruit developing period.

Estimated carbon fixations by leaf and bract throughout the whole growth period indicated that total carbon fixation by basal leaves or bracts strongly depended on their size (r = 0.95, P < 0.0001 both for basal leaf and bract). Intact and bud-removal plants fixed significantly more carbon than the vegetative plants reflecting the difference in leaf size (Table 1-3). There was no significant difference in carbon fixation between intact and bud-removal plants irrespective of difference in leaf longevity. Total carbon fixation by bracts did not differ significantly between intact and bud-removal plants (Table 1-3). Carbon fixation by bracts occupied approximately 20% of total carbon fixation per plant.

The contribution of leaf photosynthetic products to mass increment (calculated as fixed-carbon × 1/0.45 × 0.7) was estimated as 413 mg, 353 mg, and 253 mg in the reproductive-intact, bud-removal, and vegetative plants, respectively. Also, the contribution of bract photosynthetic products to mass increment was 96 mg and 85 mg in the reproductive-intact and bud-removal plants, respectively. Because total fruits mass per plants in late May was 95 ± 8.6 mg in this population (*unpublished data*, Appendix 3), the photosynthetic products of bracts largely supported the mass increment of fruits. In contrast, total carbon fixation by the photosynthesis of fruits during the fruit-developing period was –0.09 mg C, corresponding to 0.14 mg mass reduction. Therefore, the fruit photosynthetic carbon gain was sufficient to largely support respiration but not sufficient for own mass development.

^{13}C allocation

The percentage of excess ^{13}C in each organ differed between flowering and fruiting periods (Fig.1-6). At the flowering period, total excess ^{13}C per plant was 0.54 ± 0.09 mg. The ^{13}C percentage in bulbs (66%) was the highest, while that in reproductive organs was very low (< 2%) except for reproductive stems (7%). This result indicates that most of the carbon assimilated by basal leaves at the flowering season was transferred to bulbs and translocation to reproductive organs was negligible. At the late fruiting period, however, total excess ^{13}C per plant was only 0.049 ± 0.017 mg and most ^{13}C (96%) remained in leaves with little translocation to bulbs (4%). Very low C-fixation ability in this period might reflect low photosynthetic activity.

Bulb growth between years

In 2009, mean bulb volume did not differ between reproductive-intact and bud-removal plants (Table 1-4). In contrast, bulb volume was significantly larger in bud-

removal plants after the experiment. Bulbs of the bud-removal plants increased 12% after one year, while intact plants maintained almost the same bulb volume after flower and fruit production. This indicates that seed production did not limit the vegetative growth but the absence of flower and fruit production could increase the vegetative growth.

Seed production

Plants with three flowers could set an average of 1.57 ± 0.16 fruits (52.2 ± 9.2% in fruit-set rate). Both clipping treatments did not influence the fruit-set rates. In contrast, the clipping treatments significantly affected the seed set per plant (Table 1-5); average seed-set rate was 14.5 ± 3.9% for the intact, 14.6 ± 3.3% for the leaf-clipping, and 8.9 ± 2.3% for the bract-clipping treatments. Bracts clipping significantly reduced seed production, while the leaf-clipping plants produced similar number of seeds as intact plants. This result indicates the significant contribution of bract photosynthesis to seed production.

1.4. Discussion

Responses of seed production to the leaf- and bract-clipping treatments demonstrated that photosynthetic products by bracts had substantial effect on reproductive output in *G. lutea* as reported in other plant species (Wullsehleger and Oosterhuis 1990; Hori and Tsuge 1993). It is known that green parts of reproductive structures occasionally have high assimilatory capacity comparable to leaves (reviewed in Aschan and Pfanz 2003). Dry weight analysis of the carbon fixation revealed that bract's photosynthesis was able to support fruit development, but photosynthetic carbon gain by fruits

probably offset the high respiration loss. The bract-clipping treatment significantly reduced the seed production but not completely. Because the bract clipping was performed soon after flowering period, photosynthetic products by bracts during pre-flowering and flowering periods (almost half of growing season) might be stored in scape that can be used for subsequent fruit development (Lapointe 1998). Considering the similar seed-set rates between intact and leaf-clipping treatments, the current photosynthetic products by reproductive structures are assumed to be essential for seed production. Also in the ^{13}C-trace experiment, photosynthetic products by leaves were mainly transported to bulbs and the translocation to reproductive organs was negligible. These indicate that carbon resource for seed production are independent of current leaf photosynthesis at least when bracts are intact.

Allocation of resources between vegetative and reproductive plants clearly reflected their respective statuses. Vegetative plants invested first in the rapid production of shoots i.e., leaves using previously stored resources (Rothstein and Zak 2001, Ida and Kudo 2008). After shoot establishment, expansion of leaf follows, allowing efficient assimilation under bright condition before canopy closure. By the middle of May, belowground bulb already refilled for the next season. Hence, total biomass increased as the season progressed (Fig.1-2a). In the reproductive individuals, carbon investment in each organ is adjusted across the season based on their needs. Bulb mass decreased from bud to flowering stage reflecting the dependence of shoot production on stored resources. Aboveground shoot however developed rapidly from bud to flowering stage. Although the total biomass increased from flowering to early fruiting stage only bulb and flower stem increased significantly at this stage bract and leaf did not change (Fig.1-2b). Therefore the transition of flower to fruit and the refilling of bulb were very active at this period. Nevertheless, leaf and bract increased

significantly in the middle-fruiting period indicating the development of bract for seed production and leaf for the replenishment of the bulb. These results indicate the different allocation strategies in reproductive and vegetative plants of this species.

Photosynthesis by reproductive structure has been found in every reproductive part (Aschan and Pfanz 2003), and it may occupy a significant fraction of total carbon and energy provision for reproduction (Bazzaz *et al*. 1979; Hori and Tsuge 1993; Marcelis and Hofman-Eijer 1995; Antlfinger and Wendel 1997). Previous review papers (Watson and Casper 1984; Obeso 2002) reported that the contribution of non-foliar photosynthesis to reproduction varied in a broad range (from < 1% to > 60%). If resources provided by belowground storage organs contribute to current reproduction to some extent, however, contribution of non-foliar photosynthesis might be overestimated. In contrast, contribution of reproductive structures to seed production in *G. lutea* was much larger and enough to supply the resources for total fruit development. Because previously stored resources in the old tissue of bulbs have almost been exhausted by the end of flowering period (Fig. I b), furthermore, resource supply from the storage organs for fruit development may be insignificant if resource transportation from the current tissue of bulbs to fruits does not occur (we discuss this possibility later). Thus, the photosynthesis by own reproductive structures largely supports the carbon economy in seed production independent of vegetative structures in this species.

Annual bulb growth was enhanced by the suppression of fruit production by the floral-bud removal. Traditionally, the cost of reproduction hypothesis (Obeso 2002) expects that a decline in resource translocation to storage function with increasing current reproductive activity should be interpreted as a demographic cost of seed production. Specifically, cost of reproduction is defined as losses in the potential

future reproductive events, mortality, growth, and/or vegetative propagation by current reproduction. In contrast, the gross cost of seed production in *G. lutea* was largely supported by own reproductive structures, indicating the functional separation between annual seed production and bulb growth as a storage function. In other words, photosynthetic carbon translocation from leaf to new bulb was assured irrespective of reproductive investment. Therefore, increment in bulb growth in bud-removal plants might be attained by a carryover of resource, which was prepared to compensate for current seed production but was saved from expenditure. Since the bud-removal plants retained bracts as long as intact plants (Fig.1-3a), photosynthetic products by bracts should have been transported to other organs than fruits in the bud-removal plants. Thus, the compensative mechanism by own reproductive structures can mitigate a linkage between gross cost of seed production and demographic cost.

Although spring ephemerals commonly show high fruit production (Kudo et al. 2008), carbon source for fruit production varies among species, such as foliar photosynthesis early in the season (Ida and Kudo 2008), stored carbohydrates in stems (Lapointe 1998), photosynthesis by fruits (Horibata *et al*. 2007), and stored resources in old tissues (Kudo and Ida 2010). The present study newly revealed that seed production in *G. lutea* is largely supported by bract photosynthesis independent of foliar photosynthesis. The separation of carbon sources between fruit production and bulb growth may be determined by the morphological structure of bulbs (Fig. Ib). In the middle of growth period, bulb is composed of 'previous tissue' that has been almost exhausted and 'current tissue' in which current photosynthetic carbohydrates have been stored. A reproductive shoot is connected to the previous tissue, while a basal leaf is to the current tissue. This promotes the functional separation between reproductive and vegetative structure, i.e., foliar photosynthetic products are directly

stored for next season, while fruit development is self supported within the reproductive stem that is isolated from the current foliar photosynthesis. This functional separation confirms the conclusion that non-foliar photosynthesis may mitigate the cost of reproduction even after full seed production by hand-pollination.

Table 1-1. Maximum leaf size and bract size (cm^2) among treatments. Mean ± S.E., n = 20. Results of one-way ANOVA and t-test are also shown

Reproductive intact	Bud-removal	Vegetative	Statistical scores
Leaf size			
23.2 ± 1.7^a	20.4 ± 1.2^a	15.9 ± 0.8^b	$F_{2, 56} = 8.92$, $P < 0.001$
Bract size			
4.0 ± 0.3	3.7 ± 0.3		$t_{38} = 0.83$, $P = 0.41$

a, *b*: Tukey's honest significant difference test ($P < 0.05$)

Table 1-2. Result of GLM for P_{max} among treatments (reproductive intact, bud-removal, vegetative) and growth stages (pre-flowering, flowering, early fruting)

Variables	Coeffecient	s.e.	t value	P value
Intercept*	3.066	0.107	28.55	<0.0001
Treatment: bud-removal	–0.018	0.126	–0.14	0.89
Treatment: vegetative	–0.171	0.107	–1.59	0.13
Stage: flowering	0.187	0.126	1.48	0.15
Stage: fruiting	–0.977	0.126	–7.76	<0.0001

* Intercept (Treatmet: reproductive intact, Stage: pre-flowering)

Table 1-3. Estimated total carbon fixation (mg C) by leaf and bract per plant of reproductive intact, bud-removal and vegetative plants throughout the growth period in 2010. Mean ± S.E., n = 20. Results of one-way ANOVA and t-test are also shown

Reproductive intact	Bud-removal	Vegetative	Statistical scores
Leaf			
265.4 ± 18.7^a	226.9 ± 12.9^a	162.8 ± 8.7^b	$F_{2,57} = 14.8$, $P < 0.001$
Bract			
61.5 ± 5.0	54.9 ± 4.0		$t_{38} = 1.03$, $P = 0.31$

a, *b*: Tukey's honest significant difference test following one-way ANOVA ($P < 0.05$)

Table 1-4. Comparison of bulb size (cm^3) betwen reproductive intact and bud-removal treatments before and after experiment using Student t-test. Mean ± S.E.

Reproductive intact	Bud-removal	Statistical scores
Initial bulb size		
1.74 ± 0.11	1.81 ± 0.2	$t_{27} = 0.34$, $P = 0.73$
Final bulb size		
1.73 ± 0.11	2.14 ± 0.16	$t_{27} = 2.18$, $P = 0.03$

Table 1-5. Result of GLM for fruit-set and seed-set rates among leaf clipping treatments (intact, leaf-removal, bract-removal)

Variables	Coeffecient	S.E.	*z* value	*P* value
Fruit-set rate				
Intercept (intact)	-4.49×10^{-16}	0.258	-1.74×10^{-15}	1.00
Leaf-removal	0.365	0.368	0.95	0.36
Bract-removal	–0.067	0.365	–0.18	0.85
Seed-set rate				
Intercept (intact)	–1.835	0.078	–23.36	< 0.0001
Leaf-removal	0.044	0.111	0.40	0.69
Bract-removal	–0.570	0.127	–4.50	<0.0001

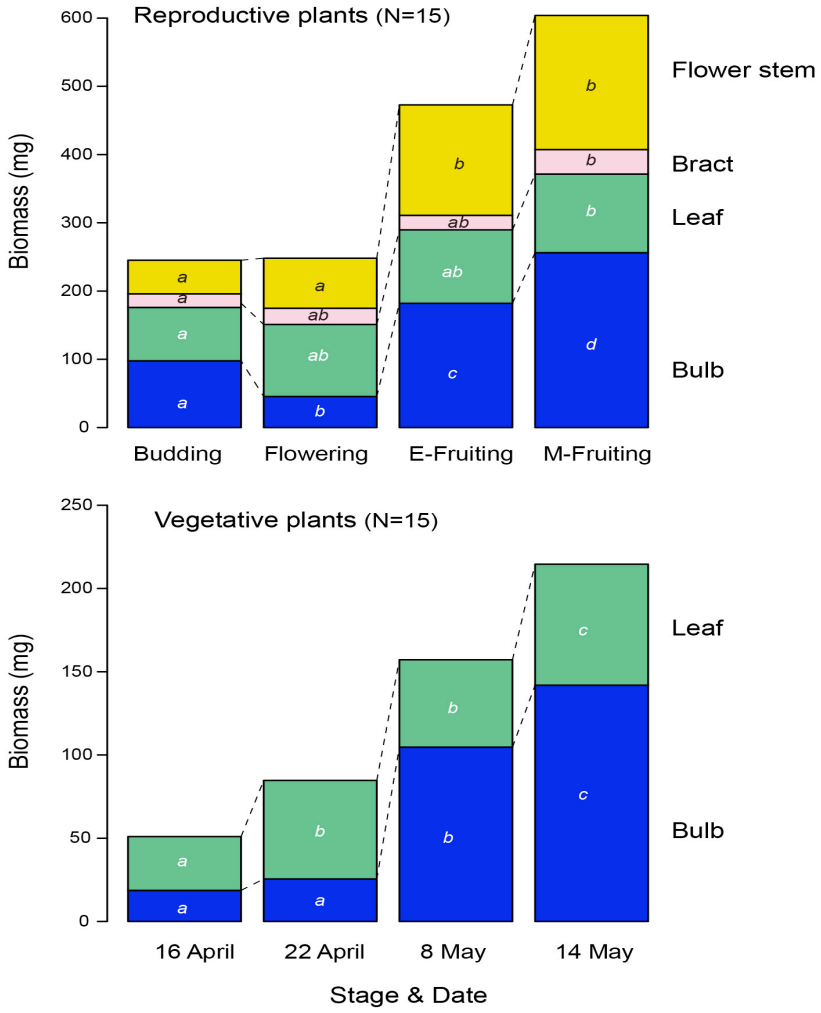

Fig. 1-1. Mean biomass of individual organ in reproductive (top) and vegetative (below) individuals across the season.

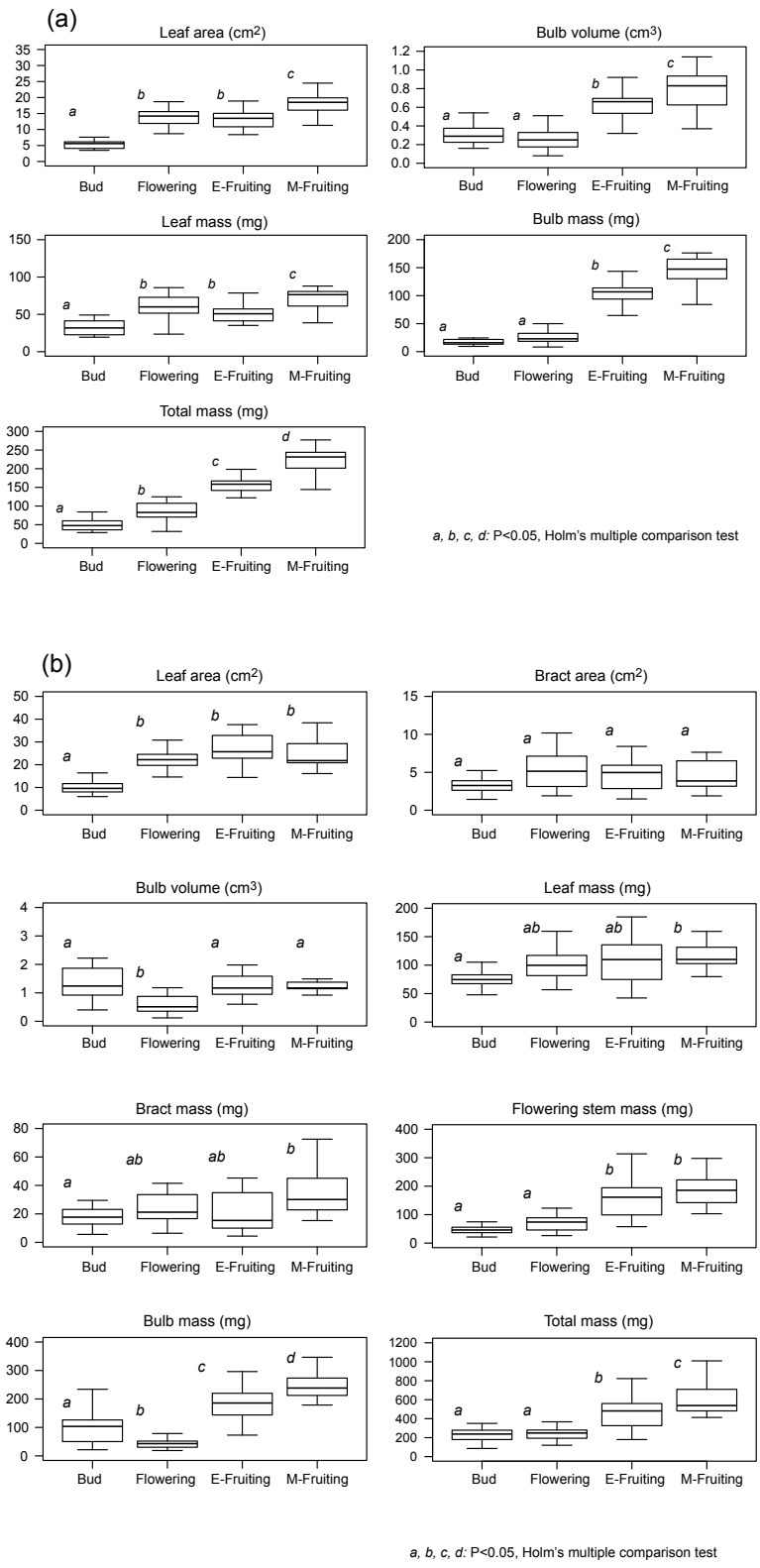

Fig. 1-2. (a) Seasonal patterns of leaf area and mass, bulb volume and mass and total plant mass across the season in vegetative plants. (b) Seasonal patterns of leaf area and mass, bract area and mass, bulb volume and mass, flowering stem and total plant mass across the season in reproductive plants.

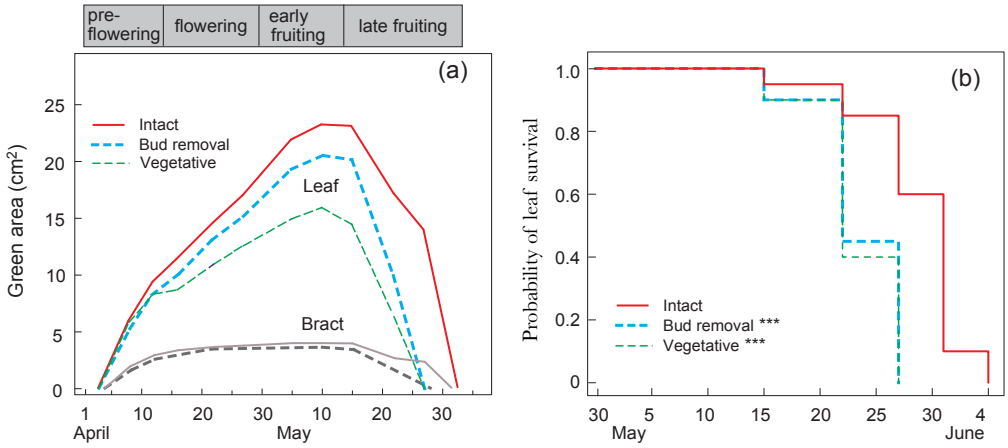

Fig. 1-3. (a) Seasonal changes in the photosynthetically active (green) area of basal leaves and bracts in reproductive intact, floral-bud removal and vegetative plants throughout the growth season. (b) Survival curves of basal leaves in intact, bud-removal and vegetative plants under natural conditions. *** P <0.0001, z = 4.20 and 4.35 for bud-removal and vegetative plants, respectively by Cox proportional hazard regression compared with intact plants.

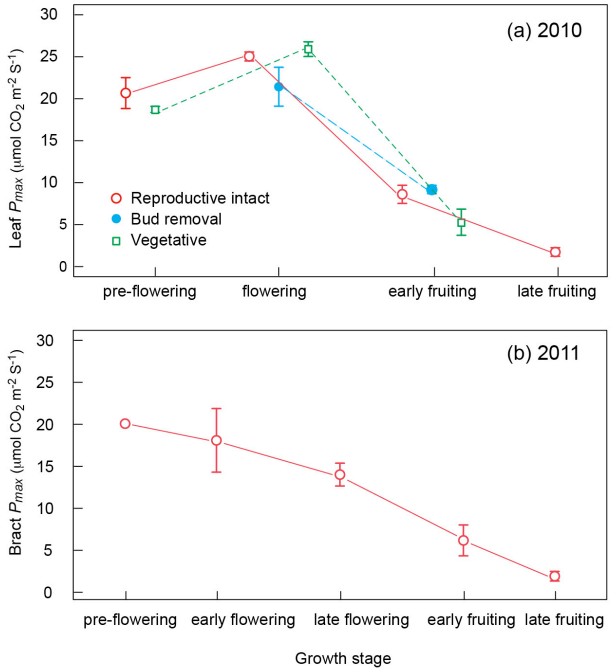

Fig. 1-4. (a) Seasonal changes in P_{max} of basal leaves in intact, bud-removal, and vegetative plants at the pre-flowering, flowering, early-fruiting, and late-fruiting stages in 2010. (b) Seasonal changes in P_{max} of bracts in 2011 at the pre-flowering, early-flowering, late-flowering, early-fruiting, and late-fruiting stages. Vertical bars indicate standard errors (S.E.).

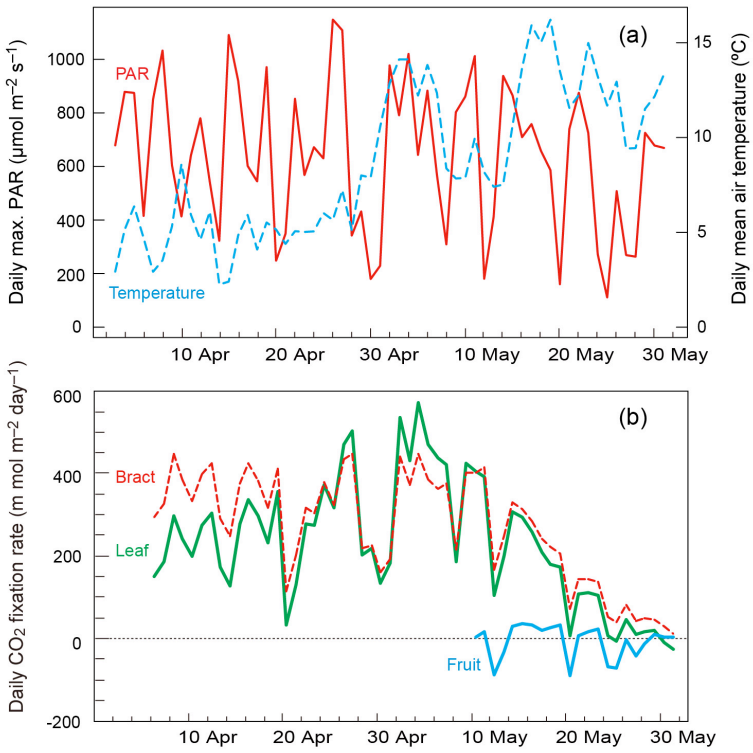

Fig. 1-5. Seasonal transitions of daily CO_2 fixation of leaf, bract and fruit estimated from actual light intensity and photosynthetic parameters of individual organs.

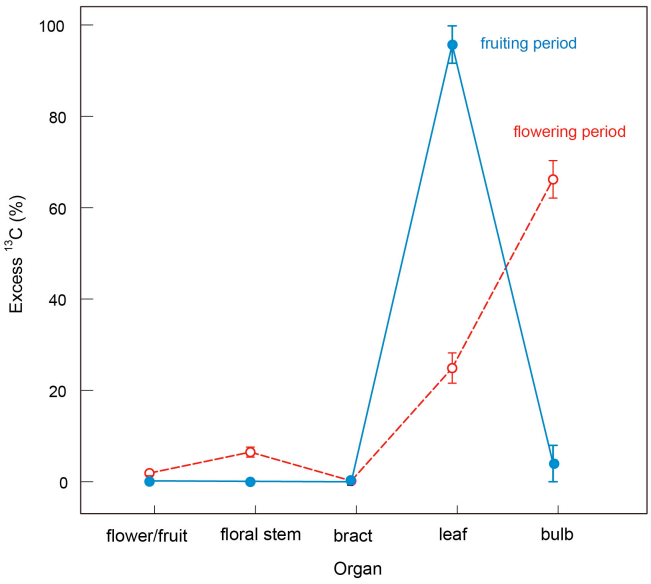

Fig. 1-6. Distribution of excess ^{13}C (%) fixed by basal leaves to individual organ at flowering (n = 9) and late fruiting (n = 4) stages. Vertical bars indicate S.E.

Appendix 1-1. Photosynthetic parameters of *Gagea lutea* estimated for basal leaves at individual growth stages and reproductive treatments (reproductive intact, vegetative, and floral-bud removal plants) in 2010

Treatment	Intact				Bud removal			
Photosynthetic parameter	α	P_{max}	R_d	θ	α	P_{max}	R_d	θ
Pre-flowering (16 April)								
Reproductive	0.051	20.68	1.81	0.54				
Vegetative	0.053	18.73	1.72	0.30				
Flowering (5 May)								
Reproductive	0.065	25.05	1.00	0.40	0.053	21.43	1.00	0.68
Vegetative	0.060	25.87	0.99	0.64				
Early fruiting (18 May)								
Reproductive	0.045	8.62	1.17	0.68	0.038	9.17	0.81	0.85
Vegetative	0.047	5.29	1.21	0.29				
Late fruiting (30 May)								
Reproductive	0.049	1.74	0.94	0.25				

Appendix 1-2. Photosynthetic parameters of *Gagea lutea* estimated for reproductive organs (fruit and bract) at individual growth stages in 2010 (for fruits) and 2011 (for bracts)

Organ	Bract				Fruit			
Photosynthetic parameters	α	$P_{\max}$	R_d	θ	α	$P_{\max}$	R_d	θ
Pre-flowering (14 April)								
	0.073	20.08	1.06	0.26				
Early flowering (28 April)								
	0.068	18.09	0.89	0.26				
Late flowering (11 May)								
	0.070	14.01	0.86	0.74				
Early fruiting (20 May)					(18 May)			
	0.057	6.17	0.41	0.31	0.043	6.44	2.67	0.82
Late fruiting (31 May)					(30 May)			
	0.062	1.91	0.92	0.86	0.145	5.56	2.49	0.09

Appendix 1-3. Dry weight allocation to individual organs at flowering (1 May) and fruiting (28 May) stages in *Gagea lutea*. Mean ± S.E.

	Leaf	Flower/Fruit	Bract	Stem	Bulb	Root	Total
Flowering stage (*n* = 14)							
Dry wt (mg)	94.6 ± 9.3	41.0 ± 4.8	20.3 ± 3.0	55.3 ± 8.3	146.6 ± 15.4	8.8 ± 1.2	366.5 ± 36.3
Percent	25.8	11.2	5.5	15.1	40.0	2.4	
Fruiting stage (*n* = 12)							
Dry wt (mg)	149.0 ± 14.7	95.0 ± 8.6	21.1 ± 3.2	72.5 ± 10.0	518.4 ± 50.9	5.0 ± 0.9	861.0 ± 77.4
Percent	17.3	11.0	2.5	8.4	60.2	0.6	

Chapter 2.

Source-sink Balance in the Reproduction and Vegetative Growth under Different Light Conditions

2.1. Introduction

Spring ephemerals assimilate carbon soon after snowmelt under high irradiance prior to canopy closure in the forest (Lapointe 2001; Rothstein and Zak 2001). This assimilated carbon may be directed towards vegetative growth and reproduction simultaneously. According to the resource allocation theory (Harper 1977; Watson 1984), there is a trade-off relationship in carbon allocation between reproduction and growth when they depend on same C-pool that is known as the cost of reproduction (Obeso 2002). Therefore, physiological interaction between vegetative growth and reproduction are important in the study of plants life history and resource use strategy.

In perennial plants, current photosynthesis and/or stored carbohydrate provide the resources needed for reproduction (Chapin *et al*. 1990) but the extent of contribution by each source can vary among species and even within species temporally (Ida and Kudo 2008; Kudo and Ida 2010). Species with a brief photosynthetic period and/or low resource availability often depend on storage for reproduction (Wardlaw 1990). Timing of leaf senescence in spring ephemeral species corresponds with the forest leaf display period and occurs 50-60 days after shoot emergence. Thus, overall carbon gain during the growing season may be limited thereby increasing the trade-offs between vegetative growth and reproduction. In addition, if present resource demand by reproductive sink surpasses current gain by

assimilative source, reproductive function may compete with vegetative function for limited resource use. However, if sink and source organ can operate independently, reproduction and vegetative growth may not compete for resource use. Therefore, detail investigation of source and sink activity is necessary to understand the extent of trade-offs between reproduction and vegetative functions.

Gagea lutea has two sources (leaf and bract) versus two sinks (fruit and bulb) in terms of carbon assimilation during a growth period (Fig. I). In Chapter 1, I reported the compensative ability of bracts when photosynthetic products by bracts were used for current seed production. On the other hand, photosynthetic products by leaves were used for bulb development and carbon translocation to reproductive function was negligible. This indicated the different source functions between leaf and bract. However, detail understanding of the interaction between sink (vegetative growth and reproduction) and source functions (photosynthesis by leaves and bracts) is yet to be ascertained. Therefore, the purpose of this chapter is to clarify the extent of trade-offs between reproduction and vegetative functions by monitoring relationships between these organs. A previous study in this species reported a high seed set success at the forest edge than under the forest canopy (Nishikawa 2009). This result gives a clue that low light condition under the canopy is likely detrimental to plants fitness but more extensive analysis is needed on this conclusion. Hence, I performed this experiment in open and under forest condition to clarify if any, the effect of decreasing light condition under the forest on the sink-source abilities.

With this experiment, I can explain the sink-source relationships between reproductive output (seed production) and vegetative growth (bulb growth) of spring ephemerals under different light conditions. To regulate the sink-source balance of whole plants, I compared the performance of reproductive intact (control), bract

removal (source reduction for reproduction), and floral-bud removal plants (removal of reproductive sink) under forest and open conditions. The following predictions are addressed in this chapter; (i) Carbon fixation by bracts will be decreased by the floral-bud removal if photosynthetic activity of bracts is sink limited, while carbon fixation by leaves may be independent of the treatments because of the fixed carbon translocation to the bulb. (ii) Vegetative growth (bulb size increment) will increase as following sequence: floral-bud removal > intact > bract removal, responding to the flexible carbon translocation by bracts, and this trend will be more clear in the open habitat if bulb growth is source limited. (iii) Reproductive output (seed-set success) will be higher in the intact plants than in the bract-removal counterparts with more seeds in the open habitat if seed production is source limited.

2.2. Methods

Experimental design

This study was conducted with potted plants of *G. lutea* from the secondary deciduous forest in the campus of Hokkaido University in 2011. Prior to the experiment, bulbs were dug from this forest floor on 12 November 2010 and taken to the laboratory for the size measurement. Width (W) and length (L) of individual bulbs were measured with a digital caliper and the volume was estimated as $4\pi \times W^2 \times L / 3$ based on the shape of the bulb. This was taken as the initial size for each plant. After the measurement, they were planted in pots (7.5 cm diameter and 10 cm depth) one by one with numbered tags for identification. Then, the pots were taken back to the forest, put in holes in order for the plant to be at the same depth as they normally grow. The floor of the forest was covered with snow from mid-December to early April.

After snowmelt in the next spring (7 April 2011), pots with reproductive plants (n = 78) were selected soon after shoot emergency and divided into two sets of three treatments; intact control (n = 13), bract removal (n = 13), and floral-bud removal (n = 13). Floral buds or bracts were removed with a forceps as soon as they appeared. All flowers of control and bract removal plants were hand-pollinated to maximize the seed production. Pollen donors for a hand-pollination were selected from multiple reproductive plants >5 m apart from the recipient plants. The floral-bud and bract removal treatments were compared with the control plants to detect the contribution of each organ to the renewal of the underground part, carbon fixation (by leaf and bract), and seed production (only bract removal and control). All plants were monitored under the forest but just before canopy closure, one set of plants was transferred to an open site (18 May 2011). This was done to reveal the effect of continuous (at open site) rather than decreasing light level experienced in the forest floor (at forest site) on plant performance. Plants were watered sometimes to prevent soil desiccation. Photosynthetically active radiation (PAR) at 2 m above the ground was automatically recorded at 1-min intervals using a combined data logger with a solar radiation sensor (HOBO weather station; Onset Co., MA, USA) during the growing period (Fig.2-1).

Individual bulb size (final bulb size) was measured once again on 21 October 2011 and the responses of final bulb volume to treatments (control, bract removal, and floral-bud removal) and light level (forest and open sites) were analyzed after the one-year experiment.

Photosynthetic carbon gain

The measurements of leaf and bract photosynthesis were conducted using natural

plants in the forest to reduce the risk of accidental damage of potted plants. Carbon fixation by leaf and bract was evaluated based on the census of leaf or bract growth, seasonal transition of photosynthetic activity, and ambient solar radiation (details available in chapter 1). Seasonal changes in leaf and bract sizes were recorded by measuring length (L) and width (W) of all plants at both sites with a digital calliper at 10-day intervals except on rainy days throughout the growth period. Basal leaf and bract area (A) was estimated as $A = 0.83 \times L \times W$ ($r^2 = 0.968$, $n = 5$) and daily changes in leaf and bract areas were quantified throughout the season. Leaf life duration of each plant was monitored until leaf senescence at the end of the season.

With a portable LI-6400 photosynthesis system (Li-Cor, Lincoln, NB, USA), photosynthetic rates of leaves per unit area were measured in three individuals randomly selected in the natural population for intact and the bud-removed plants at each of four growth stages: floral-bud stage (mid-April), flowering stage (late April), early-fruiting stage (mid-May), and late-fruiting stage (late May) in 2010. Similar measurement was performed on bracts in 2011 but only in intact plants. Removal of floral buds was performed seven days before the photosynthetic measurement. The measurement was performed under 10 light conditions (2000, 1500, 1000, 800, 500, 300, 100, 50, 10, and 0 μmol m^{-2} s^{-1}) of PAR. Leaf temperature was set at 15^{o}C and the humidity of incoming air during the measurement was maintained at 1.1 vapor pressure deficit (VPD, kPa) at an ambient CO_2 concentration of 380 μmol mol^{-1}. The net photosynthetic rate as a function of photon irradiance was expressed using a non-rectangular hyperbola (Marshall and Biscoe 1980), which is a decelerating function composed of four photosynthetic parameters; light-saturated photosynthetic rate, initial slope, degree of curvature, and dark respiration rate. Data obtained for individual plants were fitted to this equation by non-linear least-squares estimates of

the parameters. Mean values of individual parameters obtained in each period were used as representative of photosynthetic capacity. Because the width of basal leaves and bracts was smaller than the chamber size (2 × 3 cm), I corrected photosynthetic parameters by the replacement of chamber area by actual leaf area that was included in the chamber.

Net photosynthetic rates per hour were estimated from hourly mean values of light availability and photosynthetic parameters obtained at each growth stage (i.e., floral-bud, flowering, early fruiting, and late fruiting stages). Next, daily carbon fixation (mg C) was calculated from the daily average photosynthetic rate; the average value of hourly net photosynthetic rates in each day (24 hours), multiplied by leaf or bract size in each time, then total carbon fixation (mg C) throughout the growth period was estimated by summing up the daily carbon gain till the end of growing season (determined by leaf life duration). In this estimation, it is assumed that the daily transitions of leaf and bract sizes correspond to the proportional increase or decrease in sizes between days of measurements. Total assimilated carbon throughout the season by leaves and bracts was compared among treatments (intact control, floral-bud removal, and bract removal plants) between sites (open and under canopy) at individual plant level to investigate how the effect of treatments on the carbon fixation capacity is related to change in light level. Two caveats are however associated with this estimation. First, data from intact plants were used to estimate the carbon gain by the bract-removal treatment, as photosynthetic measurement was not performed on this treatment. Second, photosynthetic parameters from the natural plants were used to calculate the carbon fixation in the transferred plants. Keeping these in mind, there is possibility of under estimation of carbon fixation in the bract-removal group and the transferred plants.

Seed production

To clarify the effect of treatment and light condition on the reproductive output, infructescences from the intact and bract-removal plants from both open and forest sites were harvested before the opening of capsules. The numbers of mature seeds, aborted seeds and unfertilized ovules were counted for individual fruits. Then, the effect of treatments and light level on seed-set success (seed/ovule ratio) was assessed.

Statistical analysis

All data analyses were done using an open source system, R version 3.0.1 (R Development Core Team 2013) and best-fit models were selected for individual GLMs based on the Akaike's information criterion (AIC). To compare the maximum leaf and bract sizes among treatments at the two sites, I performed analysis of covariance (ANCOVA) in which initial bulb size (2010) was included as a covariate after log-transformation. Shapiro-Wilk test and Bartlett test were used to confirm normality and homogeneity of variances of data respectively before conducting ANCOVA. Final bulb sizes (2011) were compared among treatments between sites using a generalized linear model (GLM) postulated gamma error distribution with log-link function in which treatment and site were set as explanatory variables and initial bulb size (2010) of individual plant was included as an offset term after log-transformation. Total leaf and bract carbon fixations were analyzed using GLM postulated gamma error distribution with log-link function in which treatment and site were set as explanatory variables, and initial bulb size was included as an offset term after log-transformation to eliminate any size effect because significant size effects were detected in leaf and bract size (see results). I applied the same GLM model to

the comparisons of leaf and bract longevities in which treatment and site were set as explanatory variables. Seed-set (seed/ovule ratio) were compared between intact and bract removal treatment by GLM postulated a binomial error distribution with logit-link function in which treatment and site were set as explanatory variables.

2.3. Results

Leaf and bract longevities and carbon fixations

The lifespan of basal leaves was longer at the open site than at the forest site (t = 2.94, P = 0.0044) but the difference was less than 2 days (49.4 ± 0.2 and 51.2 ± 0.8 S.E. days in intact plants at the forest and open sites, respectively, n = 13). Although floral-bud removed plants tended to have longer lifespan (t = 2.49, P = 0.015), the differences between intact and bud removal treatments were rather small (2.3 and 0.9 days differences at the forest and open sites, respectively). Maximum leaf size was 18.89 ± 0.54 cm^2 (mean ± S.E., n = 78), and there was no difference between sites ($F_{1,72}$ = 0.20, P = 0.65) and among treatments ($F_{2,72}$ = 1.47, P = 0.24). Leaf size was strongly bulb size dependent ($F_{1,72}$ = 37.44, P < 0.001).

The lifespan of bracts was 46.8 ± 0.8 and 48.3 ± 1.2 days at the forest and open sites, respectively. In the GLM conducted for bract longevity, both site and treatment effects were not selected by AIC. Therefore, photosynthetic periods of bracts were independent of light level and the existence of fruits. Maximum bract size was smaller at the open site ($F_{1,47}$ = 4.77, P = 0.034), and significantly larger in control plants (8.72 ± 0.90 cm^2, n = 25) than in bud-removed plants (5.74 ± 0.35 cm^2, n = 26; $F_{1,47}$ = 18.95, P < 0.001). This result indicated that bract size decreased when reproductive sink was removed. Bract size was also strongly bulb size dependent ($F_{1,47}$ = 42.45, P

< 0.001).

Estimated total carbon fixation by leaves did not differ between sites and among treatments as both site and treatment effects were not selected by AIC (Table 2-1, Fig.2-2a). Estimated total carbon fixation by bracts was also similar between sites, but significantly smaller in the bud-removed plants (Table 2-1, Fig.2-2b). Furthermore, a significant interaction between treatment and site was detected for the bract carbon fixation. This was because a decrease in bract carbon fixation by the floral-bud removal was apparent only at the forest site (Fig.2-2b).

Seed production

Light level as well as the bract removal treatment clearly influenced the seed-set success (Fig.2-3). Seed-set was enhanced under bright condition and the bract removal treatment significantly reduced the seed production (Table 2-2). Bract-removed plants decreased the seed set by 53% at the forest site and 43% at the open site. This result indicates the importance of bract photosynthesis for seed production.

Bulb growth

Bulb size after one year was influenced only by treatment but not by site as site effect was not selected by AIC (Table 2-3). This result indicates that vegetative growth was independent of light levels and the bract removal treatment did not affect bulb size significantly. However, the floral-bud removal treatment significantly increased the bulb size, indicating that the failure of seed production increases the vegetative growth (Fig.2-4).

2.4. Discussion

Is carbon fixation sink-limited?

Total carbon fixation by bracts was decreased by the bud-removal treatment at the forest site. This result supports my prediction that the assimilative activity of bracts is sink-limited. Furthermore, final bract size was reduced by the bud-removal treatment at both sites, suggesting that bract growth might depend on the existence of reproductive sink (i.e., flowers). On the other hand, both leaf size and total carbon fixation were not influenced by the site and treatment as expected. This means that leaf carbon fixation simply depends on the sink intensity of bulb because most foliar photosynthetic products are transported to the bulb in this species (refer Chapter 1).

Extension of bright period did not increase the total carbon assimilation in this experiment. Although the longevities of leaves and bracts were extended at the open site to some extent, their responses were relatively conservative. Previous studies demonstrated that vegetative growth of spring ephemerals was terminated by the increase in soil temperature because warm temperature activated the metabolic process of aging (Badri *et al*. 2007; Yoshie 2008). These studies suggest that growing season length of spring ephemerals may be determined by temperature regime but not by light environment. The time of transfer, i.e., initiation of canopy closure coincided with the maximum leaf size period, and leaf size and photosynthetic activity tended to decrease after that due to the progress of partial leaf senescence (previous chapter). Therefore, delay of canopy closure may not translate into additional carbon fixation in *G. lutea* as shown by other spring ephemerals (Gutjahr and Lapointe 2008; Gandin *et al*. 2011).

Is vegetative growth source-limited?

The bud-removed plants developed bigger bulbs compared to the control and bract-removed plants, indicating that photosynthetic products by bracts could be transferred to the bulb when fruits are absent. Furthermore, the absence of significant difference in bulb size between the control and bract-removed plants indicates that bract photosynthesis basically contributes to seed production and there is no interference effect between seed production and bulb growth in this species as predicted in the first chapter.

Final bulb size did not increase at the open site irrespective of the continuous bright condition during the growth period, indicating that bulb growth is not source-limited. This is probably because bulb growth had been largely completed by the initiation of canopy closure in the forest before the transfer, and additional foliar photosynthesis did not contribute to further bulb growth as known in other forest herbs (Lapointe 2001; Gutjahr and Lapointe 2008). Furthermore, warm soil temperature at the open site could have limited the growth of bulb. Photosynthesis and stomatal conductance of spring ephemeral plants grown under warm conditions tended to decrease rapidly in late season in comparison with plants grown under cool conditions (Gandin *et al*. 2011). Such rapid decrease in the supply of photosynthetic products might inhibit further growth of bulbs. Fixed leaf phenology in response to light environment under forest may reflect the growth schedule of the spring ephemerals. Such a conservative growth response even under light rich conditions may occur when plant growth is sink-limited (Paul and Foyer 2001; Woodward 2002).

Alternative respiratory pathways might increase under enhanced light level and/or temperature. For example, activities of cyanide-insensitive respiratory pathway increased in tulip (Kanneworff and van der Plas 1994) and iris bulbs (Marissen *et al*.

1991) in response to environmental changes. Furthermore, numerous other species have shown increased respiratory loss in the underground bulbs under elevated CO_2, as a means to prevent starch buildup in the leaf (Lambers 1982; Azcón-Bieto *et al*. 1983; Gutjahr and Lapointe 2008; Kawano *et al*. 1978) and subsequent feed-back inhibition of photosynthesis (Paul and Foyer 2001; Rolland *et al*. 2002), which may trigger earlier leaf senescence and smaller final bulb size. Although I did not measure the respiration of bulb in this experiment, it was obvious that bulb growth of *G. lutea* was independent of the extension of bright period.

Is seed production source-limited?

The seed production increased significantly at the open site but decreased by the bract-removal treatment as predicted. This indicates that seed production of *G. lutea* is source-limited. In the previous chapter, I estimated that photosynthetic products by bracts could fully support the fruit development. On the other hand, some seeds were produced even in the bract-removed plants in our experiment. As a possible cause, carbon assimilation stored in the bulbs might be used for fruit development as reported in other spring ephemerals (Kudo and Ida 2010). Bulbs of *G. lutea* are composed of previous tissue and newly developing tissue at flowering stage: flowering shoot is connected to the old tissue and basal leaf is connected to the new tissue (Fig. I).

Enhanced seed production at the open site might not be due to the increased carbon assimilation by bracts because estimated carbon fixation did not differ between the forest and open sites. Self-assimilation ability of young fruits may be related to the higher seed production at the open site because green parts of reproductive structures occasionally have high assimilatory capacity comparable to

leaves (reviewed in Aschan and Pfanz 2003). Chapter one revealed that green fruits of *G. lutea* maintained relatively high photosynthetic ability until late fruiting period. Fruit photosynthesis could compensate the respiratory loss but it was insufficient for self-growth in the forest due to increasing shading in the late season. At the open site, however, a positive carbon gain might be possible under bright conditions, resulting in the acceleration of seed production.

Table 2-1. Results of GLMs for total carbon fixation by basal leaves and bracts among treatments (intact, bract-removal, bud-removal) and sites (forest, open). Only variables selected by AIC are shown

Variables	Coefficient	S.E.	*t* value	*P* value
Basal leaf*				
Intercept	4.996	0.036	138.3	<0.0001
Bract				
Intercept**	4.549	0.088	51.33	<0.0001
Bud-removal	-0.461	0.125	-3.68	0.0006
Open site	-0.224	0.125	-1.79	0.080
Bud-rem × Open	0.450	0.177	2.54	0.014

*For leaf carbon fixation, effects of treatment and site were not selected by AIC
**Intercept (treatment=intact, site=forest)

Table 2-2. Result of GLM for seed-set success per plant between treatments (intact, bract-removal) and sites (forest, open). Only variables selected by AIC are shown

Variables	Coefficient	S.E.	*t* value	*P* value
Intercept*	0.95	0.061	15.60	<0.0001
Bract-removal	-1.18	0.068	-17.35	<0.0001
Open site	0.240	0.068	3.57	<0.001

*Intercept (treatment=intact, site=forest). Interaction between treatment and site was not selected by AIC.

Table 2-3. Result of GLM for bulb size among treatments (intact, bract-removal, bud-removal) and sites (forest, open). Only variables selected by AIC are shown

Variables	Coefficient	S.E.	*t* value	*P* value
Intercept*	0.0002	0.069	0.003	0.99
Bract-removal	0.069	0.099	0.69	0.50
Bud-removal	0.290	0.098	2.95	0.0042

*Intercept (treatment=intact). Site effect was not selected by AIC.

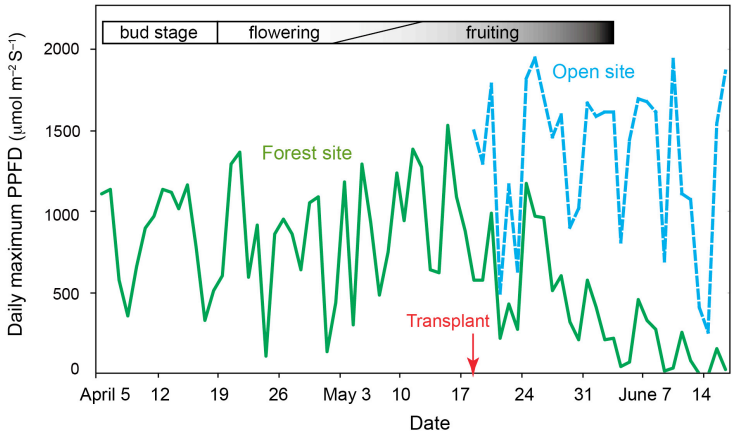

Fig. 2-1. Daily maximum photosynthetically active photon flux density (PPFD) at the forest (solid line) and open sites (broken line) during the growing season in 2011. Average periods of budding, flowering and fruiting are indicated. The transfer to open site was conducted on 18 May.

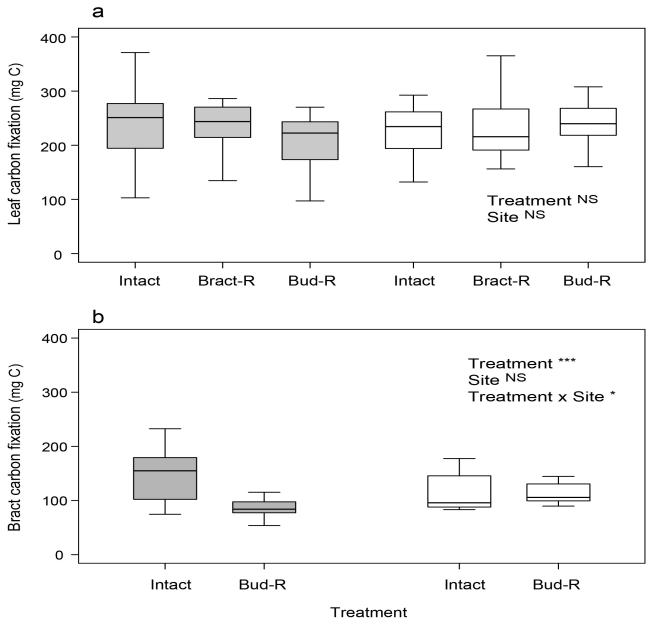

Fig. 2-2. Total carbon fixation by basal leaf (a) and bract (b) of intact, bract removal (only for a), and floral-bud removal plants throughout 2011 growth period at the forest (gray) and open sites (white). Carbon fixation values are adjusted for initial bulb size (Autumn 2010) to remove the size effect from the site and treatment effects. Box plots indicate 25, 50 and 75 percentile and whiskers indicate 10 and 90 percentile of data distribution. Statistical results by GLM are indicated (see Table 2-1 for details).

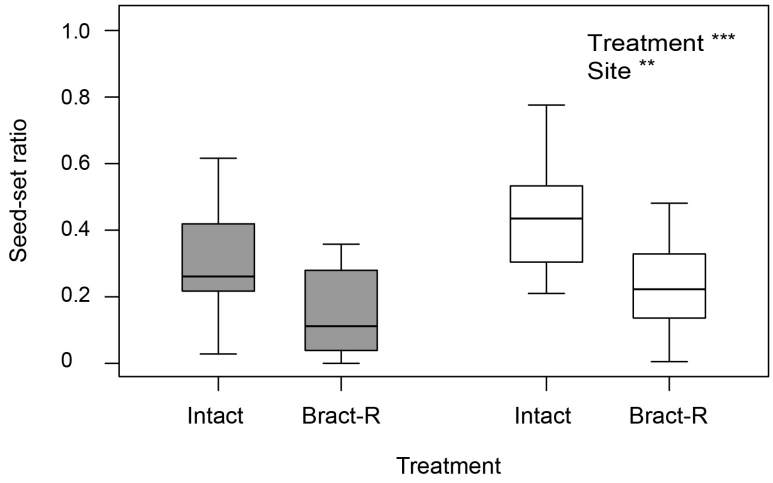

Fig. 2-3. Seed-set per plant at the forest (gray) and open sites (white) in the intact and bract removal treatments. Statistical results by GLM are indicated (see Table 2-2 for details).

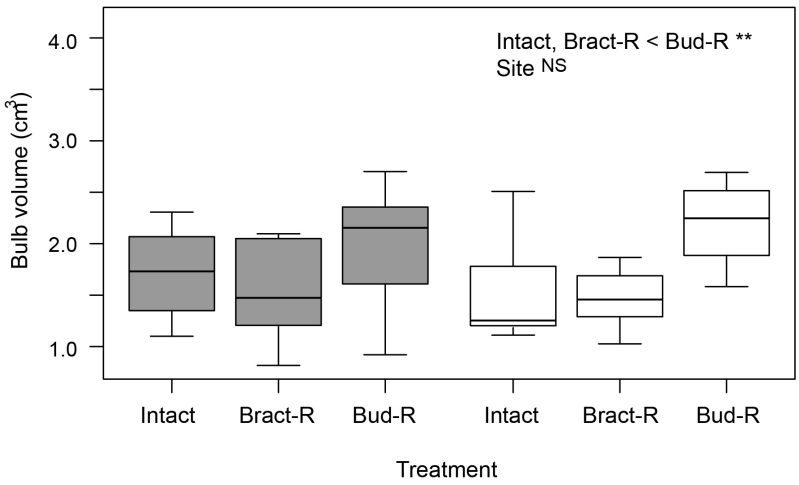

Fig. 2-4. Bulb volume after one-year experiment at the forest (gray) and open sites (white) in the intact, bract removal, and floral-bud removal treatments. Bulb volume values are adjusted for initial volume to remove the size effect from the site and treatment effects. Statistical results by GLM are indicated (see Table 2-3 for details)

Chapter 3.

Responses of Reproduction and Growth to Warming Climate

3.1. Introduction

Understanding of the responses of plant growth and reproduction to environmental variation is crucial for deciphering the relationships between plants and their environments (Granier and Tardieu 2009). In higher latitude ecosystems with clear seasonality, temperature is one of the most important environmental factors influencing plant growth. Generally, low-growth temperature tends to reduce growth rate and biomass accumulation even for plants adapted to cool environments. In spring ephemerals, however, increased growth and larger storage organs are evident at lower temperature (Wheeler *et al*. 2004; Lapointe and Lerat 2006; Badri *et al*. 2007). For example, *Erythronium americanum* (Lapointe and Lerat 2006; Gandin *et al*. 2011) and *Crocus vernus* (Badri *et al*. 2007; Lundmark *et al*. 2009) developed bigger corms under cool conditions. The enhanced growth at lower temperature is correlated with extended leaf longevity due to a slower starch accumulation by the sink organ leading to reduced source–sink imbalance compared to higher temperature (Gandin *et al*. 2011).

The growth initiation of spring ephemerals depends mostly on the time of snowmelt and subsequent temperature (Fitter *et al*. 1995). Hence, future climate change that is predicted to cause the most apparent effects in early spring, should cause serious impacts on the existence of spring ephemerals. Menzel (2000) already showed that the leafing out of canopy trees has become earlier due to warmer spring temperature. The growth of spring ephemerals may also be restricted under this

scenario of earlier spring and increasing temperature early in the spring. Furthermore, an earlier closure of the canopy due to increased spring temperature (Menzel 2000, 2002) could also reduce their growth, by limiting the favorable light period on the forest floor and hence reduced photosynthetic carbon accumulation even with earlier snowmelt (Rothstein and Zak 2001). Thus, climate change impacts on spring ephemerals should be evaluated in terms of direct warming effect and indirect light conditions.

Previous studies have looked into this but most of these studies were conducted for non-reproductive individuals due to their simple whole-plant morphology (i.e., one source versus one sink; Gandin *et al*. 2011; Gutjahr and Lapointe 2008; Lapointe and Lerat 2006). To predict the response of spring ephemerals to climate change, however, the responses of reproductive plants should be clarified. Sensitivity to thermal environment may vary between reproductive and vegetative plants reflecting the specific carbon allocation strategy (Chapter 2).

Reproductive plants of *G. lutea* have two source (leaf and bract) versus two sink functions (fruit and bulb) in terms of carbon assimilation during a growth period (Chapter 1 and 2). Leaves and bracts acted as specialized source organs for bulb growth and current seed production, respectively, but photosynthetic products by bracts could be flexibly used for bulb growth when plants failed to set fruits. Hence, monitoring the reproductive individuals of *G. lutea* under warming conditions could help us clarify whether irrespective of reproductive status their growth is also limited at high temperatures as found in the non-reproductive counterparts. In this chapter, I explored the hypothesis that the extent of reproduction and growth may differ under warming conditions depending on individual source organ for each trait (i.e., leaf and

bract). If leaf and bract lifespan and carbon gain are not restricted under warmer temperature, seed production and bulb growth may not suffer under climate change.

In addition to the source-sink balance for resource allocation, warmer temperature may directly influence the pre-zygote process, i.e., pollination and fertilization success, such as pollen viability, pollen tube growth, stigma receptivity, and ovule viability, which may also decrease seed production of plants under warm climate (Hedhly *et al*. 2008). Especially, pollen activity is generally sensitive to temperature (Hedhly *et al*. 2005; Kakani *et al*. 2005). Therefore, thermal responses of pollen germination rates may also influence reproductive success under warm climate.

Linking data for environmental factors, physiological and phenological responses of leaves and bracts (source function), reproductive activities, and bulb growth among forest, open and greenhouse conditions, I investigated the responses of reproductive performance and vegetative growth to earlier and warmer spring in *G. lutea*. In this experiment, I aim to predict the warm-spring impacts on spring ephemerals by separation of temperature (greenhouse vs. open habitat) and light effects (open vs. forest habitat). I expect that the responses of reproductive performance (seed production) and vegetative performance (bulb growth) may vary reflecting the separate source functions and resource demand of sink functions as clarified in Chapter 2.

3.2. Methods

Experimental design and growth conditions

Bulbs of *Gagea lutea* were collected in December 2013. They were immediately taken to the laboratory and their volumes were measured as mentioned before.

Individual bulbs of similar sizes were then planted in pots with numbered tags for identification, and the pots were randomly transferred to three plots: forest and two open plots outside the forest. In March of 2014, I advanced snowmelt in spring at one of the open plots by manually removing snow twice (20th and 26th March). Then I set a greenhouse over the plot to facilitate rapid natural snowmelt of the remaining snow and increase the temperature. Hence, I established three plots in this study; forest (intact conditions), open (continuously bright but same snowmelt time with the forest site), and greenhouse (GH) plots (continuously bright and warm with early snowmelt). To generate the snowmelt dates between the forest and open plots, I added 50 to 70 cm of snow to the forest plots because snow depth was deeper at the open plot in comparison with the forest plot. Preliminary growth conditions were characterized by monitoring soil temperature before the experiment to check the differences in thermal conditions among plots. Six automatic data loggers, two per plot (HOBO, UA-002, Onset Computer Corporation, Bourne, MA), were randomly set up in pots for measuring soil temperature at the depth of 10 cm at 1-h intervals from December 2012 to June 2013. Data obtained by two loggers at each plot were averaged. Air temperature (at every plot) and photosynthetically active radiation (PAR; at the forest and open plots) were recorded during the experimental period at 1-h intervals using a combined data logger with a solar radiation and thermometer (HOBO weather station; Onset Co., MA, USA) from 7 March to 5 June 2014. Averages of 24 measurements within 1 day were stored as daily means.

Physiological measurements of leaves and bracts

To investigate the physiological responses of leaves and bracts to environmental manipulations among sites, leaf and bract maximum photosynthesis, P_{max} at saturation

irradiance (1000 μmol m^{-2} s^{-1}) and dark respiration were measured using a portable LI-6400 photosynthesis system (Li-Cor, Lincoln, NE, USA). Three of the experimental plants were selected per plot at each of three growth stages: 7th April at floral-bud stage, 16th April at flowering stage and 8th May at early fruiting stage at the GH plot; 18th April, 12th May and 23rd May at the forest plot; and 18th April, 10th May and 24th May at the open plot, respectively. Respiration rate was measured after leaving the leaf for 5-7 min in the dark (at 0 μmol m^{-2} s^{-1} irradiance). Leaf temperature in the chamber was controlled at 20°C, and the concentration of CO_2 in ambient air entering the leaf chamber was maintained at 380 μmol mol^{-1}. Leaf-to-air vapor pressure deficit (VPD) was controlled to be less or equal to 1.1 kPa.

After shoot emergence in early spring, all reproductive plants were monitored. To assess the seasonal changes in photosynthetic area, the leaf and bract sizes (length, *L* and width, *W*) of all plants were measured with a digital caliper on a weekly basis but as soon as senescence started, monitoring and measurement changed to every other day till the end of growth period. This measurement involved only the green area that was photosynthetically active. Leaf and bract area (*A*) was estimated as $A = 0.83 \times L \times W$ ($r^2 = 0.968$, $n = 5$). After removing damaged plants, 45, 49 and 40 plants were present at the forest, open, and GH plots, respectively.

Pollen germination and reproductive output

To test the effect of temperature on pollen germination activity of *G. lutea*, *in vitro* pollen germination experiment was performed at three different temperatures in the laboratory. First, agar-based media composed of suitable sucrose concentration (ca. 10%) was prepared in a test tube. Next, flowers with fresh and dehisced anthers were collected from a nearby *G. lutea* population and brought immediately to the

laboratory. Two drops of the agar-based media were placed on each of 15 glass slides placed on a wet filter paper in a petridish. One glass slides contain two fields so that there are 30 fields in all. Uniform pollen grain samples were dispersed vertically on each media field and the slides were immediately incubated at each of 10, 20, and 30°C temperature, respectively, at 80% humidity for 18 hours. The number of germinated pollen grains was then counted under the microscope.

To evaluate the reproductive output under varying environmental conditions, the number of floral buds was recorded in each plant at every plot during the flowering period. Then, hand pollination was conducted for every flower to eliminate the pollen limitation for seed production. To prevent inbreeding depression, pollen donors for the hand pollination were selected at least 5 m away at the forest and open plots. Because flowering in the GH occurred earlier than natural conditions due to early snowmelt, however, hand pollination at the GH plot was conducted between pots. Soon before seed dispersal, all infructescences were harvested. In the laboratory, individual fruits were opened carefully and the numbers of mature seeds and undeveloped ovules were counted in each fruit. Fruit-set ratio was expressed as matured fruit number divided by original flower number, and seed production was taken as the ratio of mature seed number to original ovule number produced per plant. Duration of flowering period was also recorded at all plots to clarify any environmental effects on flowering phenology.

Bulb growth

Annual bulb growth was measured to clarify the responses of perennial organ (i.e., vegetative growth) to environmental variations among plots. Initial bulb sizes were measured in autumn of 2013 (see above). Final bulb sizes were measured again on

20th June 2014 after the growth period. Bulb growth was taken as the final bulb volume after one growth season in response to plot differences and initial bulb size.

Statistical analysis

Leaf and bract maximum photosynthesis, P_{max} and dark respiration were analyzed using generalized linear model (GLM) postulated gamma error distribution with log-link function in which plot (forest, open, GH) and growth stage (budding, flowering and fruiting) were set as explanatory variables. In this GLM, open plot and budding stage were included in an interception term. I conducted the same GLM for the comparison of final bulb size (2014) in which plot was set as an explanatory variable and initial bulb size (2013) was set as an offset variable after log-transformation. . Pollen germination rate was compared by GLM postulated binomial error distribution with logit-link function in which temperature was set as an explanatory variable. I analyzed flower, fruit and mature seed numbers using GLM postulated Poisson error distribution with log-link function, while fruit-set success (fruit/flower ratio) and seed-set success (seed/ovule ratio) were compared by GLM postulated binomial error distribution in which plot was set as an explanatory variable. Leaf and bract survival rates were compared among sites using the Cox proportional hazards regression model. Maximum leaf and bract sizes among sites were compared by the analysis of covariance (ANCOVA) in which initial bulb size (2013) was included as a covariate after log-transformation. Turkey's HSD test was used for post hoc multiple comparisons. All statistical analyses were conducted using an open source system, R version 3.0.1 (R Development Core Team 2013) and best-fit models were selected for individual GLMs based on the Akaike's information criterion (AIC).

3.3. Results

Growth conditions

In the preliminary measurement of soil temperature in 2013, soil conditions were constantly maintained around 0-1ºC during the winter (December to February) at every plot, indicating no frost soil. Mean soil temperature during the growth season of *G. lutea* (April and May) was 7.3°C (ranging from 0.1 to 20.2ºC) at the forest plot. Daily mean soil temperatures at the GH and open plots were 4.3ºC and 0.6ºC warmer than the forest plot, respectively (data is not shown).

Air temperature during the experimental period in 2014 showed similar trend to soil temperature in 2013. Mean air temperature throughout the growth season (April and May) was 10.2°C (ranging from -1.2 to 28.0ºC) at the forest plot. Daily mean temperatures at the GH and open plots were 3.5ºC and 0.8ºC warmer than the forest plot during the growth season, respectively (Fig.3-1a). Daily maximum temperatures at the GH and open plots were 15.3ºC and 3.1ºC warmer than the forest plot, respectively. PAR at the open plot was two times larger than that at the forest plot (Fig.3-1b). As the season progressed, the difference in PAR between open and forest habitats became larger due to developing canopy closure in the forest. Therefore distinct growth conditions characterize each plot.

Physiological traits of leaves and bracts

Leaf P_{max} showed no difference among plots but significant difference among growth stages (Table 3-1). P_{max} at bud and flowering stages were significantly higher than that at fruiting, indicating the decreasing physiological activities in late growth period. Furthermore, a significant interaction occurred between GH and fruiting stage due to

rapid decrease in P_{max} at the GH plot (Fig.3-2a). This indicates a rapid decrease in physiological activity under warm conditions.

The seasonal trend of dark respiration rates differed between the GH and other two plots (Table 3-2). Leaf respiration rates generally decreased as the season progressed at the open and forest plots but remained high at the GH plot, especially at fruiting stage leading to a significant interaction between GH and fruiting stage (Fig.3-2b). Therefore, the GH condition encouraged high respiratory loss compared to the open and forest conditions.

Contrary to leaf P_{max}, bract P_{max} was significantly different among plots. They were higher at the open and GH plots than at the forest plot corresponding to the irradiance conditions (Fig.3-3a). Bract P_{max} was also different among growth stages in which values at fruiting stage were significantly lower than at bud and flowering stages. Similar to leaf P_{max}, however, bract P_{max} was also very low at fruiting at the GH plot leading to an interaction between GH and fruiting stage.

Bract dark respiration rates changed seasonally but not different among plots (Fig.3-3b). Although bract respiration decreased as stage progressed at the open and forest plots, it increased greatly at fruiting at the GH plot, indicating that warmer temperature encouraged more respiratory loss also for bracts.

Leaf as well as bract life span varied significantly among plots in this experiment (Fig.3-4). Initiation of leaf senescence began after 25, 34, and 34 days at the GH, open, and forest plots, respectively; mean leaf longevity was 43.3 ± 0.5, 49.4 ± 0.5, and 50.3 ± 0.4 S.E days at the GH, open and forest, respectively. Mean bract longevity was 42.1 ± 0.5, 48.8 ± 0.5, and 52.6 ± 0.4 days at the GH, open and forest plots, respectively. Leaf longevity was significantly shortened at the GH plot ($P < 0.001$) but no difference between open and forest plots ($P = 0.365$). Bract longevity

was shortened at the open and GH plots compared to the forest plot as shown by Cox proportional hazard regression ($P < 0.001$, Fig.3-4).

Maximum leaf and bract sizes also showed significant differences among plots. Plants at the GH plot produced significantly smaller leaves and bracts compared to the plants at the open and forest plots (Table 3-3).

Reproductive activity

Acceleration of snowmelt at the GH plot advanced both the shoot growth initiation and reproductive phenology (Fig.3-5). Although flowering duration was similar among plots (14, 15 and17 days at the open, forest and GH plots, respectively), flowering started earlier at the GH plot (8 days after shoot emergence) compared to the forest and open plots (16 days after emergence).

Mean flower number was similar between the open and GH plots, but lower at the forest plot (Table 3-4). On the contrary, fruit number, total seed number, fruit-set success, and seed-set success were all significantly lower at the GH plot, while they were similar between the forest and open plots (Table 3-4).

Mean pollen germination rate was 23.9%, 33.2%, and 7.9% at 10°C, 20°C, and 30ºC, respectively (Fig.3-6). The highest germination rate was recorded at the intermediate temperature ($z = 3.84$, $P < 0.001$). The highest temperature, which mimiced the GH condition, significantly inhibited pollen germination ($z = -6.84$, $P < 0.0001$). These results indicate that warm conditions during growth season might cause serious effects on fertilization success and subsequent seed production in *G. lutea*.

Bulb growth

Although initial bulb volume was not different among plots (Fig.3-7a), final bulb volume was significantly smaller at the GH plot but larger at the forest plot in comparison with the open plot (Fig.3-7b). Change in bulb size (difference between final and initial volume) also reflected an increase at the forest plot but a decrease at the GH (Fig.3-7c). Bulb size increased by 26% at the forest plot, but it showed 33% decrease at the GH plot. Therefore, warmer temperature had a negative effect on the bulb growth.

3.4. Discussion

Physiological responses of leaves and bracts

As reported previously, warming by the GH caused earlier snowmelt, increased air and soil temperatures compared to the open and forest plots (Shaw and Harte 2001), while light availability were similarly high at the open and GH plots compared to the forest plot. These environmental variables influenced leaf and bract characteristics in *G. lutea*. Leaf P_{max} commonly decreased at fruiting period reflecting the short lifespan of spring ephemerals' leaves. The present study clearly revealed that the decrease in leaf P_{max} was accelerated in the GH, indicating an earlier physiological aging under warm conditions. The response of bract P_{max} was little bit different from that of leaf P_{max}. Bract P_{max} was significantly lower at the forest plot in comparison with the open and GH plots during early to middle growth period. However, bract P_{max} at the GH plot was strongly suppressed at fruiting stage, while the decline of bract P_{max} at the forest plot was not apparent. Because bract photosynthesis contributes to fruit development (Chapter 1), these responses of bract photosynthetic activity should affect seed production of *G. lutea* (as discussed in later). In both leaf and bract, dark

respiration rate was largest at fruiting period especially under warm conditions, meaning that most carbon fixed at this period might be directed towards respiration loss.

A direct effect of temperature on the enzyme activity could be a possible explanation for the lower P_{max} in the GH. In addition to low physiological activities at fruiting stage, there is possibility of light water stress at the open and GH plots, which could also influence P_{max} and respiration rate (Lange *et al*. 1971). Plants at the forest and open plots had similar trend of respiration rates compared to the GH that had their respiration increasing with time. This suggests that temperature condition may be more important than light conditions for respiratory activities. High respiratory loss has been reported in other spring ephemerals grown under warm conditions (Gardin *et al*. 2011; Bernatchez and Lapointe 2012).

Phenological responses

Responses of leaf and bract phenology seem to be influenced greatly by temperature rather than light conditions, because leaf longevity was shortened at the GH plot but similar between the open and forest plots. Bract longevity was also shortened at the GH plot but extended at the forest than the open plot. Furthermore, leaf and bract sizes were smaller at the GH plot compared to the open and forest plots with similar sizes. Despite a relatively high P_{max} at the GH plot early in the season, reduced leaf longevity and sizes, in addition to high respiration loss, might reduce the overall performance under warm conditions in terms of carbon assimilation by leaf and bract. Gradual senescence process and larger leaf and bract sizes at the forest plot, however, might be useful for efficient carbon fixation with less respiratory loss. This means that individual sink organs (bulb and seed) related to these source organs (leaf and bract)

might suffer insufficient carbon supply at the GH plot. Yoshie (2008) reported an extended longevity for *Gagea lutea* under cool growth temperatures in contrast to a summer-green forb *Maianthemum dilatatum* in which cool growth temperatures shortened leaf lifespan.

Onset of flowering occurred earlier at the GH plot compared to the forest and open plots. Flowering onset in spring ephemerals highly depends on the snowmelt timing (Kudo *et al*. 2004, 2008; Thomson 2010) as found also in most alpine species (Sparks *et al*. 2000; Inouye 2008; Hoffmann and Sgro 2011). Flowering duration and flower number, however, appeared less affected by warming in this study. Menzel and Fabian (1999) reported an elongation of growing season in Europe during the last decades but flowering duration showed little or no change. Warming experiment in high altitude also documented both prolonged flowering (Dunne *et al*. 2003) and no change in flowering duration (Price and Waser 1998) in response to warming. Most species have shown a reduced flower number in early snowmelt years (Inouye 2008). That flower duration and flower number were less affected in this study also confirmed that response to warming is species specific (Menzel *et al*. 2006; Lambrecht *et al*. 2006). It could also mean that production of flower in this species is less costly and as such, warming did not limit flower production. It is known that aboveground shoot construction in early blooming forest herbs commonly depends on resources stored during the preceding year (Muller 1978; Routhier and Lapointe 2002). As the case in the first chapter, flower production also depends on previously stored resources in this species thereby making warming effect undetectable because storage organ had been filled before the warming treatment.

Reproductive responses

Contrary to flower production and despite hand pollination exercise, seed-set success was lower at the GH plot, indicating that factors other than pollen limitation affected seed production in this experiment. Generally in the absence of pollen limitation, spring ephemerals usually have high potential seed set (Schemske *et al*. 1978, Kudo *et al*. 2008) due to high photosynthetic carbon gain prior to canopy closure (Niesenbaum 1993). Although bract exhibited increased P_{max} and moderate respiration rate at bud and flowering stages, the significant reduction in P_{max} and stimulation of respiration at fruiting season might have resulted into reduced seed-set in the GH. Irrespective of pollination exercise, the rate of fruit abortion may increase if the rate of carbon assimilation is suppressed, resulting in a low fruit-set success (Stephenson 1980; Chiariello and Gulmon 1991). Since bract is responsible for seed production in this species (Chapter 1), reduced bract longevity, smaller bract size and consequently reduced bract assimilation in the GH might be responsible for the lower seed set. In addition, with less pollen germination rate at 30°C, it is possible that pollen germination rate were inhibited in the GH due to low fertilization success. Heat stress inhibited pollen vigor and stigma receptivity in some crop plants (Devasirvatham *et al*. 2012b; Kaushal *et al*. 2013).

Contrary to the results of Chapter 2 (2012), seed-set rates did not differ between open and forest plots, indicating no advantage of extended bright period for seed production in 2014. In the previous study (Nishikawa 2009), higher seed-set success was reported in plants inhabiting forest-edge habitat in comparison with plants inhabiting forest habitat. These contradictory results indicate that the advantage of longer bright condition may vary from year to year. Growth initiation of *G. lutea* under natural conditions in 2004 occurred one-week earlier due to earlier snowmelt. Thus, most reproductive plants might have completed seed production by the time of

canopy closure in this year. This indicates no light-resource limitation for seed production in early-snowmelt year.

Bulb growth responses

Bulb growth was strongly influenced by the varying environmental conditions. Plants grown at the forest plot stored more resources underground, i.e., larger final bulb volume, compared to the open and GH plots. In contrast, plants accumulated least biomass at the GH plot compared to the open and forest plots. Therefore, vegetative growth of *G. lutea* is negatively influenced by warming conditions. This result is similar to several previous reports conducted on non-reproductive spring-ephemeral species for example; *E. americanum* produced larger bulbs under lowest temperature regime (Gandin *et al*. 2011), *Allium cepa* increased bulb biomass under lower growth temperature (Daymond *et al*. 1997), and *Crocus vernus* bulb growth was stimulated under cool spring temperature (Badri *et al*. 2007). Reduced leaf lifespan, smaller leaf area and larger respiratory carbon loss could be the major explanation for smaller bulb size under warming conditions.

Bulb volume was also smaller at the open plot compared to the forest plot. This trend was contrastive to the result of Chapter 2, where the bulb size was maintained when plants were transplanted to open habitat during the second half of the growth season. As explained above for seed production, the longer exposure to open condition in this experiment could have led to high transpiration and even heat and water stress that might affect the growth of bulb.

Based on these results, cooler temperature under the forest early in the spring is beneficial for spring ephemerals, and the predicted future climate warming may be detrimental for the growth and reproduction of spring ephemerals. Nevertheless, a

long-term monitoring of population dynamics is needed to evaluate the exact trend because changes in growth rate and seed-set success should affect the population dynamics of perennial plant species.

Table 3-1. Result of GLM for leaf and bract P_{max} among growth stages (budding, flowering, fruiting) at the forest, open, and GH plots.

Variables*	Coefficient	S.E.	*t* value	*P* value
Leaf				
Intercept	3.19	0.10	31.1	<0.001
Forest	−0.12	0.15	−0.83	0.41
GH	0.098	0.15	0.67	0.50
Flowering	–0.23	0.15	–1.56	0.13
Fruiting	−1.26	0.15	−8.69	<0.001
Forest × Flowering	–0.02	0.21	–0.12	0.90
GH × Flowering	0.22	0.21	1.09	0.28
Forest × Fruiting	0.17	0.21	0.84	0.41
GH × Fruiting	–0.46	0.21	–2.24	0.03
Bract				
Intercept	2.79	0.10	26.7	<0.001
Forest	–0.36	0.15	–2.40	0.021
GH	0.27	0.18	1.51	0.14
Flowering	–0.018	0.15	–0.12	0.91
Fruiting	–0.81	0.15	–5.51	<0.001
Forest × Flowering	0.06	0.21	0.31	0.76
GH × Flowering	0.09	0.23	0.39	0.68
Forest × Fruiting	0.42	0.21	2.00	0.052
GH × Fruiting	–1.41	0.26	–5.52	<0.001

* Intercept (Plot: open, Stage: Bud)

Table 3-2. Result of GLM for leaf and bract respiration rates among growth stages (budding, flowering, fruiting) at the forest, open, and GH plots

Variables*	Coefficient	S.E.	*t* value	*P* value
Leaf				
Intercept	0.96	0.09	10.24	<0.001
Forest	0.11	0.13	0.84	0.41
GH	–0.02	0.13	–0.19	0.85
Flowering	–0.37	0.13	–2.77	0.008
Fruiting	–1.02	0.13	–7.64	<0.001
Forest ×Flowering	0.07	0.19	0.37	0.71
GH ×Flowering	0.12	0.19	0.69	0.49
Forest ×Fruiting	0.28	0.19	1.50	0.14
GH ×Fruiting	1.01	0.18	5.38	<0.001
Bract				
Intercept	0.71	0.17	4.14	<0.001
Forest	–0.07	0.24	–0.30	0.76
GH	0.05	0.29	0.17	0.86
Flowering	–0.38	0.24	–1.56	0.12
Fruiting	–0.84	0.24	–3.49	0.001
Forest ×Flowering	0.39	0.34	1.14	0.26
GH ×Flowering	0.06	0.38	0.16	0.86
Forest ×Fruiting	0.34	0.34	0.98	033
GH ×Fruiting	1.24	0.42	2.93	0.006

* Intercept (Plot: open, Stage: Bud)

Table 3-3. ANCOVA results of the maximum leaf and bract size (cm^2) at the forest, open, and GH plots. Mean ± S.E.

Variable	Forest	Open	GH	Statistical scores
Leaf size	11.49 ± 0.5[a]	11.20 ± 0.46[a]	5.00 ± 0.30[b]	$F_{2, 145} = 80.22$, $P < 0.001$
Bract size	2.81 ± 0.23[a]	2.72 ± 0.12[a]	1.68 ± 0.09[b]	$F_{2, 145} = 26.95$, $P < 0.001$

[a, b] Tukey's honest significant difference test ($P < 0.05$)

Table 3-4. Flower, fruit and seed production at the forest, open, and GH plot. Mean ± S.E.

Plot	N	Flower no.	Fruit no.	Seed no.	Fruit set	Seed set
Forest	45	3.9 ± 0.2*	3.4 ± 0.2	65.0 ± 4.6***	0.89 ±0 .03	0.59 ± 0.03
Open	49	4.9 ± 0.2	4.2 ± 0.3	74.2 ± 4.0	0.83 ± 0.02	0.58 ± 0.02
GH	40	4.5 ± 0.2	3.2 ± 0.2*	55.4 ± 4.8***	0.73 ± 0.04***	0.47 ± 0.03***

Results of GLM are indicated (* $P < 0.05$; *** $P < 0.001$).

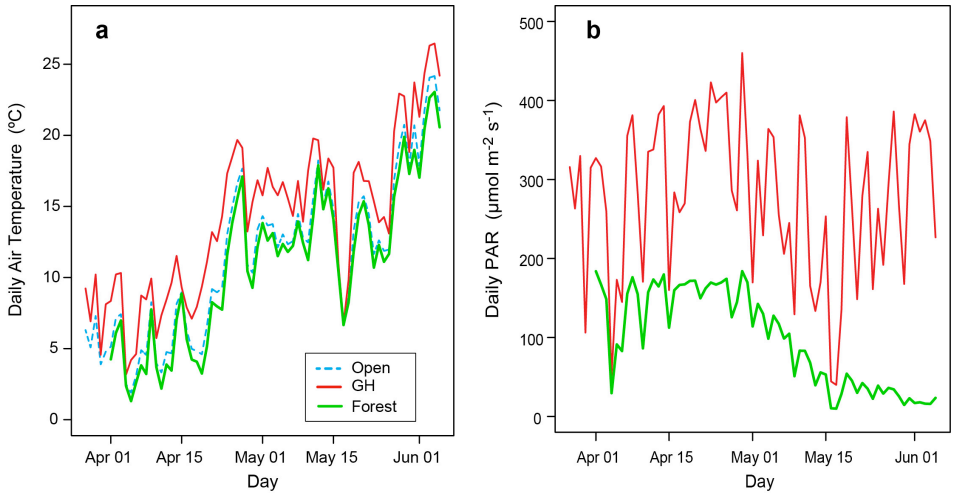

Fig. 3-1. Seasonal fluctuations in (a) air temperature and (b) photosynthetically active radiation (PAR) at the forest (green), open (blue), and GH plots (red), respectively.

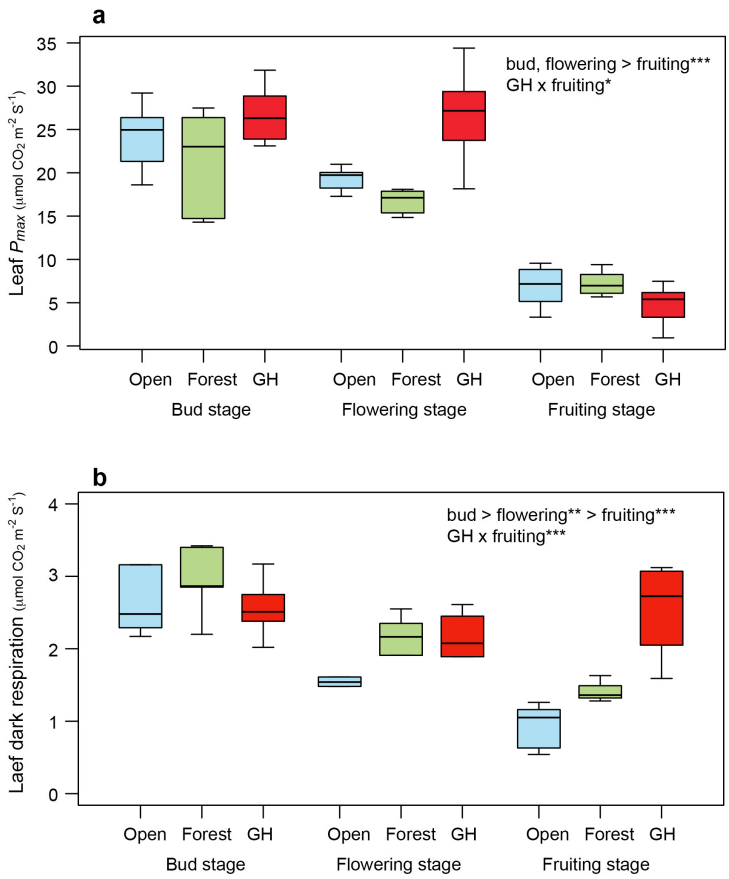

Fig. 3-2. Seasonal changes in leaf P_{max} (a) and dark respiration rates (b) at the forest (green), open (blue), and GH (red) plots at bud, flowering and fruiting stages. Results of GLM are indicated (** $P < 0.01$; *** $P < 0.001$).

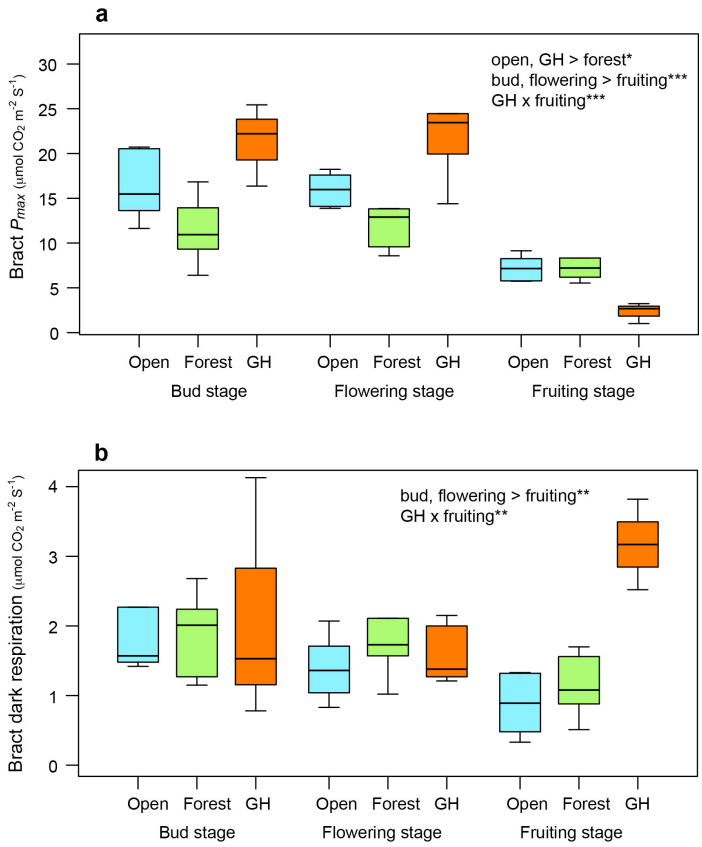

Fig. 3-3. Seasonal changes in bract P_{max} (a) and dark respiration rates (b) at the forest (green), open (blue), and GH (pink) plots at bud, flowering and fruiting stages. Results of GLM are indicated (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

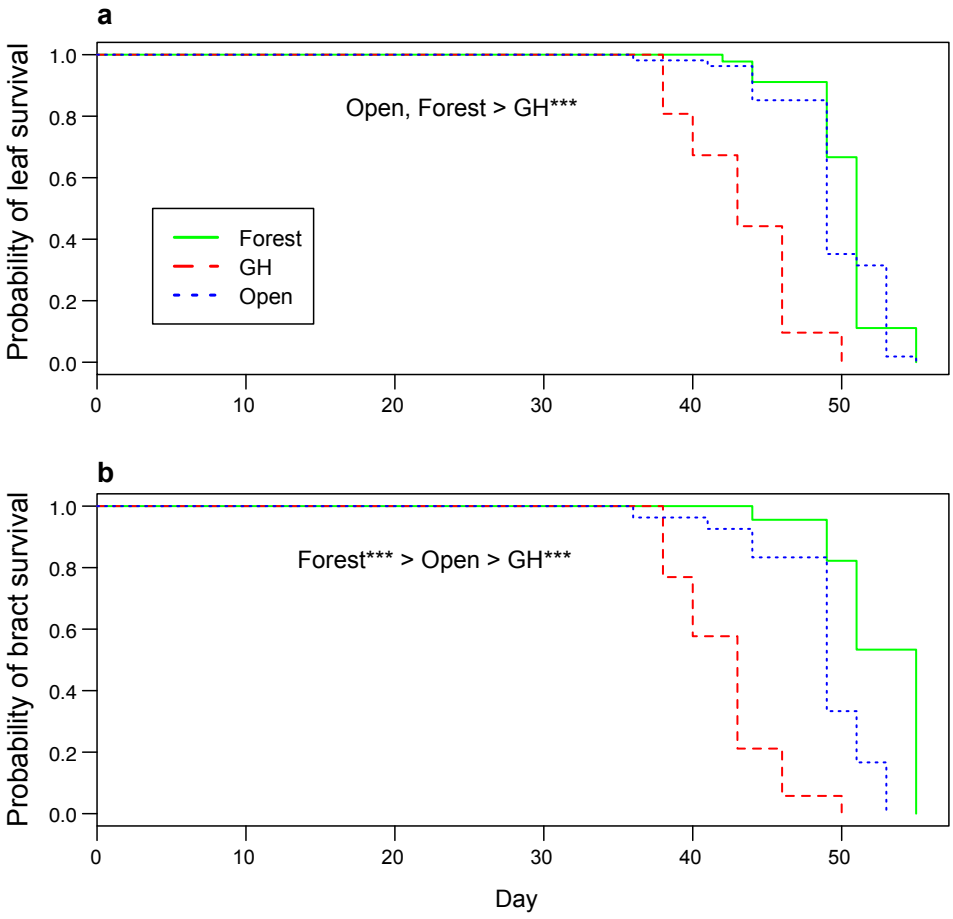

Fig. 3-4. Survival curves of leaves (a) and bracts (b) at the forest (green), open (blue), and GH (red) plots in 2014. Results of GLM are indicated (*** $P < 0.001$).

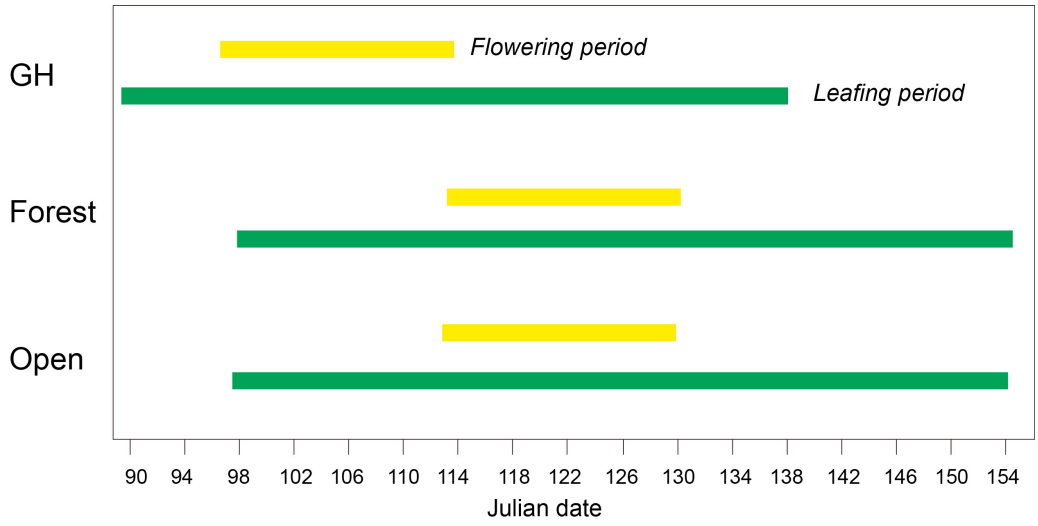

Fig. 3-5. Growing season length and flowering period at the forest, open, and GH plots in 2014.

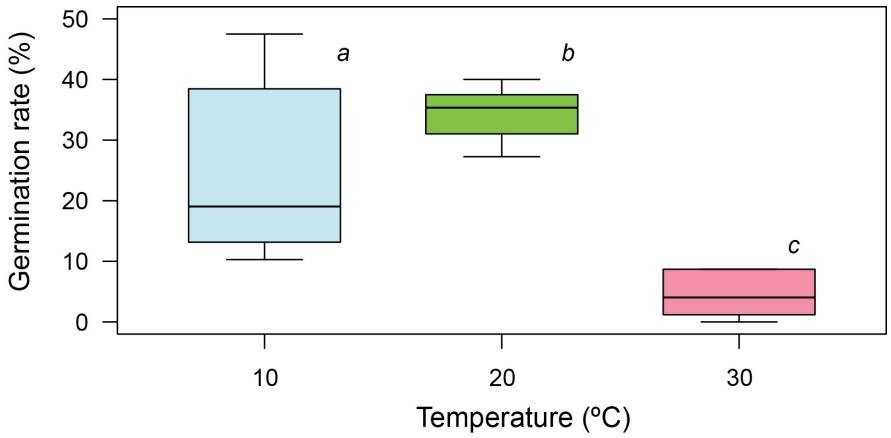

Fig. 3-6. Percentage germination rate of pollen incubated at 10°C, 20°C and 30°C. n = 30. Results of GLM are indicated (*b* > *a* > *c*, $P < 0.001$).

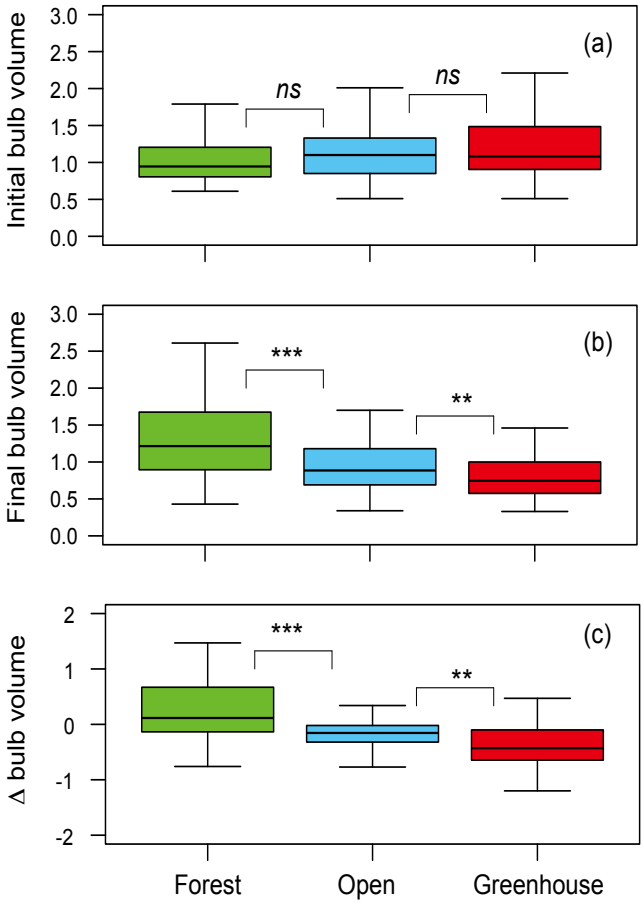

Fig. 3-7. Initial (a), final (b) and change in bulb volume after one-year (c) at the forest (green), open (blue), and GH (red) plots. Results of GLM are indicated (** $P < 0.01$, *** $P < 0.001$).

General Discussion

Reproductive compensation in spring ephemerals

Spring ephemeral plants exhibit a typical characteristic of "*sun plant*" thereby taking the advantage of the short high-irradiance period between snowmelt and canopy closure. Accumulated photosynthetic products during this period are used for reproduction and vegetative growth simultaneously. In *Gagea lutea*, reproduction and vegetative growth depend on separate source organs leading to more or less no cost of reproduction, i.e., no trade-off between seed production and bulb growth. Cost of reproduction has been defined as the losses in potential future reproductive success caused by current investment in reproduction (Obeso 2002). However, this might not always be the case if some compensatory mechanisms occur. Spring ephemerals commonly have high reproductive output (Kudo *et al*. 2008) due to various compensatory mechanisms exhibited by individual species and/or high photosynthetic assimilation under high irradiance.

This study newly revealed the allocation strategy of *G. lutea* (Chapter 1). The leafy bracts in the reproductive individuals are solely responsible for seed production, while the leaf is responsible only for vegetative growth. This division of labor between leaf and bract effectively mitigates the cost of reproduction in this species. Absence of cost of reproduction was revealed by the similar initial and final bulb sizes even after full seed production followed by a hand-pollination treatment. Furthermore, important and interesting finding is the flexible allocation of photosynthetic products by bracts depending on the reproductive success. When flower buds were removed, plants showed larger bulb formation owing to the additional carbon allocation from bracts. In general,

this clear and reasonable strategy enables this species to minimize the cost of reproduction and carry out sexual reproduction from year to year.

Species-specific compensatory mechanisms seem to be common for many spring ephemeral species. For example, fruit production in *Trillium apetalon* (Melanthiaceae) depends on current foliar photosynthesis during the fruiting period, and resource translocation to fruit is accelerated with increasing shade stress (Ida and Kudo 2008). In *Trillium erectum*, carbohydrates stored in the stem during the flowering period are the major source of fruit production (Lapointe 1998). In *Adonis ramosa* (Ranunculaceae), fruit production is independent of foliar photosynthesis but depends mostly on photosynthesis by the fruits (Horibata *et al*. 2007). In *Corydalis ambigua* (Fumariaceae), finally, nectar production at flowering stage depends on foliar photosynthetic products but seed production depends on the previously stored resources in old tissue (Kudo and Ida 2010). These various patterns of resource pool for reproduction indicate that the maintenance of high reproductive activity is crucial for spring ephemerals but there are many ways to attain that.

Sink-source balance of resource allocation

The growth and development of spring ephemerals have been believed to be sink rather than source limited because similar leaf lifespan was found in plants grown under constant light conditions as well as under natural conditions (Lapointe and Lerat 2006). Although most previous studies were conducted for non-flowering plants, it is now clear that this statement is not totally true for reproductive plants because not all aspects of their activities are sink-limited in their growth. In the present study, development of perennial organs was sink-limited, while seed production could be source-limited because they indicated higher seed production under open conditions and lower seed production

by the bract removal (Chapter 2). Although there was no difference in carbon assimilation by bracts between the forest and open sites, increase in fruit assimilation under bright condition could explain the resulting higher seed production at the open site.

Similar to previous studies, there was no increment in leaf assimilation even under open habitat, and no difference in bulb growth between forest and open habitats. Generally, plants adjust the growth of their organs in response to their photosynthetic status especially when nutrients is not limiting. Nevertheless, the lack of growth increment under light-rich (this study) or CO_2–rich conditions (e.g., Woodward 2002) may occur when plant growth is sink-limited. Sink limitation has been shown in many species, such as a typical spring ephemeral plant (*Erythronium americanum*; Gutjahr and Lapointe 2008), a bulbous plant (onion; Daymond *et al*. 1997), and a tuberous plant (potato; Conn and Cochran 2006). These results indicate that resource allocation to vegetative growth is relatively conservative, while resource investment in reproductive function may be more opportunistic.

Warming effects on spring ephemerals: implications for climate change

The warming experiment using a greenhouse (GH) clearly revealed the negative effects of warming on both growth and reproduction of spring ephemeral plants (Chapter 3). Under warm condition, P_{max} decreased and respiration rate increased sharply at fruiting period, resulting in an extensive reduction of carbon assimilation. In addition, leaf longevity was shortened and leaf size decreased as reported in the previous studies (Lapointe and Lerat 2006, Yoshie and Fukuda 1994). This translated into a smaller bulb size in the GH. Although bract indicated relatively high photosynthetic activity under open and warm conditions during bud and flowering stages, low photosynthetic activity, high respiration rate and shortened bract lifespan could explain the low seed production

in the GH. A negative effect of warm temperatures on the pollen germination rate is another possible cause of reduced seed production in the GH due to low fertilization success. However, further studies are necessary for a clarification of relative importance of photosynthetic activity and fertilization success for reproductive success under warming climate in spring ephemerals.

Forest habitat is characterized by relatively stable thermal conditions in comparison with open habitat. This encourages less respiratory loss and maintenance of high leaf and bract P_{max} for longer period that led to larger bulb development and moderate seed production. Badri *et al.* (2007) reported the impact of both air and soil temperature on the growth of *Crocus vernus*. Corm mass and cell size were larger and leaf lasted longer at cool temperature regime than at higher temperature regime. *Gagea lutea* also showed a similar response and by adding to existing information, this study also showed a decreased reproductive output under early snowmelt and warm spring. These results indicate that global warming may cause serious impacts on both reproductive and vegetative aspects of spring ephemerals.

Spring ephemerals have unique and species-specific resource allocation strategies to maintain reproductive output and replacement of storage organs even under fluctuating environmental conditions in the deciduous forests. In the case of extended light availability, effects on spring ephemerals may depend on other environmental factors, such as temperature and soil moisture. Under warm conditions, however, both reproductive and vegetative aspects are negatively affected. Nevertheless, effects of warming temperature on the reproductive allocation may vary among species having different resource pool. Therefore, monitoring of multiple spring ephemeral species with different resource pool for seed production is needed to reach a perfect conclusion on the effect of the predicted spring temperatures on the reproduction of spring ephemerals.

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