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Title	Warm temperature conditions restrict the sexual reproduction and vegetative growth of the spring ephemeral Gagea lutea (Liliaceae)
Author(s)	Sunmonu, Ninuola; Kudo, Gaku
Citation	Plant Ecology, 216(10), 1419-1431 https://doi.org/10.1007/s11258-015-0520-6
Issue Date	2015-10
Doc URL	https://hdl.handle.net/2115/63014
Rights	Authors may self-archive the author's accepted manuscript of their articles on their own websites. Authors may also deposit this version of the article in any repository, provided it is only made publicly available 12 months after official publication or later.
Type	journal article
File Information	Sunmonu&Kudo2015PlantEcol.pdf

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3   Warm temperature conditions restrict the sexual reproduction and vegetative

4   growth of the spring ephemeral *Gagea lutea* (Liliaceae)

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6   Ninuola Sunmonu[1], Gaku Kudo[1*]

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8   *[1]Faculty of Environmental Earth Science, Hokkaido University, Sapporo 060-0810,*

9   *Japan*

10   *[*]For correspondence. E-mail: gaku@ees.hokudai.ac.jp*

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12   Running title: Effect of warming on spring herbs

13

Abstract

The responses of reproduction and growth to climate warming are important issues to predict the fate of plant populations at high latitudes. Spring ephemerals inhabiting cool-temperate forests grow better under cool conditions, but how reproductive performance is influenced by warm weather is unclear. The phenological and physiological responses of reproduction and vegetative growth to warm temperature and light conditions were evaluated in the spring ephemeral *Gagea lutea*. Leaf and bract physiological activities, bulb growth, and seed production were compared among reproductive plants grown in forest, open, and greenhouse (GH; warming manipulation in the open site) plots. *In vitro* pollen germination ability was tested under various temperatures. In the GH, leaf and bract photosynthetic activities decreased rapidly at the fruiting stage, but dark respiration rates remained high, resulting in higher carbon exhaust in warm conditions. Both leaf and bract sizes and their longevities were reduced in the GH. Annual bulb growth was largest in the forest plot and smallest in the GH plot. Pollen germination was strongly inhibited at high temperature (30°C). Fruit and seed productions were decreased only in the GH plot. Both vegetative and reproductive activities were negatively affected by warm temperature, resulting in less vegetative growth and lower seed set, whereas an understory habitat was beneficial for vegetative growth and showed similar seed production to an open habitat over the experimental period. Decreasing population dynamics of spring ephemerals was predicted in response to future warming climate not only by growth inhibition but also by restriction of seed production.

Key words: leaf traits, pollen germination, reproduction, spring ephemeral, vegetative growth, warm spring.

1 **Introduction**

2

3 To predict the impact of climate change on population dynamics of plant species, it is

4 crucial to understand how plant growth and reproduction respond to environmental

5 variations (Hedhly et al. 2008). In high latitude ecosystems with clear seasonality,

6 temperature is one of the most important environmental factors influencing plant growth.

7 Generally, enzyme activity, cell division, and photosynthetic activity decrease in cool

8 temperatures in many plants (Fitter and Hay 1987; Tardieu et al. 2000). However,

9 previous studies reported that spring ephemerals, mostly perennial herbs inhabiting the

10 floor of cool-temperate deciduous forests, grow better at cool temperatures (Lapointe

11 2001; Lapointe and Lerat 2006; Badri et al. 2007; Gandin et al. 2011; Bernatchez and

12 Lapointe 2012). For example, *Erythronium americanum* (Lapointe and Lerat 2006;

13 Gandin et al. 2011) and *Crocus vernus* (Badri et al. 2007; Lundmark et al. 2009)

14 developed bigger storage organs under cool conditions. The enhanced growth under cool

15 conditions is correlated with extended leaf longevity and/or continuous sink intensity of

16 bulbs or corms in terms of carbon partitioning compared with warmer temperatures

17 (Lundmark et al. 2009; Gandin et al. 2011).

18 Initiation of growth in spring ephemerals depends mostly on the time of snowmelt

19 and subsequent temperature (Schemske et al. 1978; Fitter et al. 1995; Whigham 2004).

20 The significant variations in temperature encountered so far have been mainly recorded at

21 the end of winter and the beginning of spring (Easterling et al. 1997; Sparks et al. 2000).

22 Actually, the timing of extreme temperatures could have a significant impact on the

23 existence of spring ephemerals that emerge very early in the spring (Whigham 2004;

24 Kudo et al. 2008). Furthermore, increased spring temperature commonly advances the

25 flushing of canopy trees (Menzel 2000, 2002; Vitasse et al. 2009). Earlier closure of the

canopy could also reduce carbon assimilation by limiting the favourable light period on the forest floor and hence reduce photosynthetic carbon accumulation (Niesenbaum 1993; Rothstein and Zak 2001; Ida and Kudo 2008). Thus, the impact of climate change on spring ephemerals should be evaluated in terms of direct warming effects and indirect light conditions.

Previous studies have looked at the extent of growth of spring ephemerals in different growth temperature regimes but only for a limited range of species. In addition, probably because of the simple whole-plant morphology (i.e., one source versus one sink), most of the work has concentrated on non-reproductive individuals (Gandin et al. 2011; Gutjahr and Lapointe 2008; Lapointe and Lerat 2006; Bernatchez and Lapointe 2012). To predict the response of spring ephemerals to climate change, however, the responses of reproductive plants should be clarified because the timing and frequency of extreme temperature events could be important also for sexual reproductive phases and final reproductive output (Kudo et al. 2004). In addition, sensitivity to thermal environment may vary between reproductive and non-reproductive plants, reflecting the specific carbon allocation strategy (Sunmonu and Kudo 2014).

Gagea lutea is an ideal model plant to quantify the responses of reproduction to warming climate because reproductive individuals have two sources (leaf and bract) versus two sink functions (fruit and bulb) in terms of carbon assimilation during a growth period (see illustration in Sunmonu et al. 2013). Leaves and bracts act as specialized source organs for bulb growth and current seed production, respectively, but photosynthetic products from bracts could be flexibly used for bulb growth when plants fail to set fruits (Sunmonu et al. 2013). Therefore, by monitoring reproductive individuals of *G. lutea* in warm conditions, we could clarify whether irrespective of reproductive

status their growth is also limited at warm temperatures, as found in non-reproductive counterparts of other spring ephemerals.

In this study, we explored the hypothesis that the extent of reproductive output and bulb growth in warm conditions would depend on the responses to source organs for each sink function (i.e., bract for seed production and leaf for bulb growth). Seed production and bulb growth may not be sensitive to climate change if the lifespan and carbon assimilation of the leaf and bract are not restricted under warm conditions. Apart from the source–sink balance for resource allocation, warmer temperature may directly influence the pre-zygote process even under conditions of good pollination success, i.e., fertilization success, such as pollen viability, pollen tube growth, stigma receptivity, and ovule viability, which may also decrease seed production (Hedhly et al. 2008). Because pollen activity is generally sensitive to temperature (Hedhly et al. 2005; Kakani et al. 2005), the thermal influence on pollen performance may also influence reproductive success in a warm climate.

By linking data for environmental factors, physiological and phenological responses of leaves and bracts (source functions), reproductive activities and bulb growth (sink functions) among forest, open, and greenhouse (GH; warming manipulation in the open site) conditions, we investigated phenological and physiological responses of photosynthetic activity, reproductive performance, and vegetative growth to earlier and warmer spring in *G. lutea*. In this experiment, we intended to predict if warm spring conditions impact on spring ephemerals by separating temperature effects (GH vs. open habitat) from light effects (open vs. forest habitat). We expected that responses in terms of reproductive (fruit and seed production) and vegetative (bulb growth) performance to growth conditions would vary based on the responses of individual source organs. The objectives of this study were to clarify the effects of early and warmer spring on (a) leaf

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and bract characteristics, (b) reproductive output, and (c) bulb growth in *G. lutea*, a typical spring ephemeral species.

Materials and methods

Study species and experimental design

Gagea lutea Ker-Gawl. (Liliaceae) is a polycarpic perennial herbaceous species inhabiting northern temperate forests. This species has a typical spring ephemeral lifecycle; flowering 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). It is pollinated by insects, and cross-pollination is more effective for seed production (Kudo *et al*., 2004). Aboveground shoots usually die at the same time as seed dispersal, at the time of canopy closure in late May. Thus, the short period between snowmelt and canopy closure is when this species accumulates resources in the underground bulb. 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 (Sunmonu *et al*., 2013).

Bulbs of *G. lutea* were collected (n = 203) from a secondary deciduous forest within the campus of Hokkaido University, Sapporo, Japan (43$^{°}$ 04' 57" N, 141$^{°}$ 20' 22" E) in late autumn of 2012. This forest is usually covered with snow from early December to early April, and common canopy trees in this fragmented forest include *Ulmus davidiana* var. *japonica*, *Cercidiphyllum japonica*, *Betula platyphylla* var. *japonica* and *Populus maximowiczii*. The bulbs were immediately taken to the laboratory and their volumes were measured. Width (*W*) and length (*L*) of individual bulbs were measured using a

1 digital calliper, and the volume was estimated as $\pi \times W^2 \times L/6$ based on the shape of the

2 bulb. In this study, we used volume as an index of bulb size because of the non-

3 destructive measure. In our preliminary measurement, however, we confirmed a strong

4 correlation between bulb volume and dry mass ($r^2 = 0.837$, $n = 15$). Individual bulbs were

5 then planted in pots with numbered tags for identification, and the pots were randomly

6 transferred to three plots: forest (n = 67) and two open plots outside the forest (n = 68 in

7 each plot) . In March of 2013, advancement of snowmelt timing was performed at one of

8 the open plots by manually removing snow twice (14th and 21st March). Then we set a

9 GH over the plot to facilitate rapid natural snowmelt of the remaining snow and increase

10 the temperature. The GH was 2 m in width, 3 m in length, 2 m in height, and covered

11 with clear plastic sheet. Hence, we established three plots in this study; forest (natural

12 habitat), open (continuously bright but same snowmelt time as the forest plot), and GH

13 plot (continuously bright and warm with early snowmelt). In the first year (2013), plants

14 were made to acclimate to the environmental conditions. In late autumn of 2013 before

15 snow cover, bulb sizes from all plots were measured again and replanted in preparation

16 for the next spring. The same exercise was repeated in early spring 2014 (snow removal

17 on 20th and 26th March), but to generate similar snowmelt dates between forest and open

18 plots, we added 50 to 70 cm of snow to the forest plot because snow depth was deeper at

19 the open plot.

20

21 Growth conditions

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23 Preliminary growth conditions were characterized by monitoring soil temperature among

24 plots throughout the growth season in 2013. For this, six automatic data loggers, two per

25 plot (HOBO, UA-002, Onset Computer Corporation, Bourne, MA), were randomly set in

pots to measure soil temperature at a depth of 10 cm at 1-h intervals from December 2012 to June 2013. The data obtained by two loggers in each plot were averaged. In 2014, air temperature (in every plot) and photosynthetically active radiation (PAR; in the forest and open plots) were recorded during the experimental period at 1-h intervals using a combined data logger with a solar radiation monitor and thermometer (HOBO weather station, Onset Co., MA, USA) from 7 March to 5 June. Averages of 24 measurements within 1 day were stored as daily means. We did not measure soil temperature in 2014 because of technical difficulties.

Physiological measurements of leaves and bracts

After shoot emergence in spring 2014, all reproductive plants producing floral buds were monitored. To investigate the physiological responses of leaves and bracts to environmental manipulations among plots, leaf and bract maximum photosynthetic rates (P_{max}) at saturation irradiance (1500 μmol m^{-2} s^{-1}) and dark respiration rates 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 in the GH plot; 18th April, 12th May, and 23rd May in the forest plot; and 18th April, 10th May, and 24th May in the open plot. Respiration rate was measured after leaving the leaf for 5–7 min in the dark (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}. This temperature corresponded to the typical daytime temperature on clear days in the growth period of *G. lutea* (see results) in which photosynthetic activity is maximal (Sunmonu et al. 2013). Leaf-to-air vapor pressure

deficit (VPD) was controlled to be less than or equal to 1.1 kPa. Because the width of leaves and bracts was smaller than the chamber size (2 × 3 cm), we corrected photosynthetic parameters by the replacement of chamber area by actual leaf area that was included in the chamber. This protocol of photosynthetic measurement was determined based on our previous studies in *G. lutea* (Sunmonu et al. 2013; Sunmonu and Kudo 2014).

To assess the seasonal changes in photosynthetic area, the leaf and bract sizes (length, L and width, W) of all plants were measured using a digital calliper on a weekly basis, but as soon as senescence started, monitoring and measurement changed to every other day till the end of the 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.

Reproductive output

To evaluate the reproductive output in various environmental conditions, the number of floral buds was recorded for each plant in every plot during the flowering period. Then, artificial outcrossing by hand pollination was conducted for every flower to eliminate pollen limitation for seed production. Soon before seed dispersal, all infructescences were harvested and taken to the laboratory, where individual fruits were opened carefully and the numbers of mature seeds and undeveloped ovules in each fruit were counted. 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

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1 produced per plant. Duration of the flowering period was also recorded for all plots to

2 clarify any environmental effects on flowering phenology.

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4 Bulb growth

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6 Annual bulb growth was measured to clarify the response of perennial organs (i.e.,

7 vegetative growth) to environmental variations among plots. Initial bulb sizes were

8 measured in November 2013 (see above). Final bulb sizes were measured again in late

9 June 2014 after the growth period. Bulb growth was taken as the final bulb volume after

10 one growth season in response to plot differences and initial bulb size.

11

12 Pollen germination

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14 To test the effect of temperature on fertilization success, pollen germination ability was

15 measured at five different temperatures in the laboratory. First, agar-based media with a

16 suitable sucrose concentration (10%) was prepared in a test tube. Next, flowers with fresh

17 and dehisced anthers were collected from a nearby *G. lutea* population, placed in plastic

18 bags and brought immediately to the laboratory. Two drops of media were placed

19 separately on each of 15 glass slides kept on petri dishes lined with moist filter paper.

20 Uniform pollen grain samples were dispersed vertically on each medium field, and three

21 slides (six media) each were immediately incubated at temperatures of 10, 15, 20, 25, or

22 30°C at approximately 80% humidity for 24 hours. Pollen germination was determined

23 by direct microscopic observation (BX43, Olympus Corporation, Tokyo, Japan). Pollen

24 was considered germinated when the length of the germinated pollen tube exceeded the

25 grain's diameter. For each temperature treatment, the numbers of germinated and

1   shrunken pollen grains were counted using a microscope, and the germination percentage

2   was evaluated by dividing the number of germinated pollen grains per field of view by

3   the total number of pollen per field of view. Furthermore, the length of pollen tubes of

4   randomly selected 40–60 pollen tubes in each temperature was measured using digital

5   photographs.

6

7   Statistical analysis

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9   Leaf and bract P_{max} and dark respiration rates were analysed using generalized linear

10 models (GLMs) with a postulated gamma error distribution with log-link function, in

11 which plot (forest, open, GH) and shoot age (day from emergence) were set as

12 explanatory variables. Leaf and bract survival rates were compared among plots using

13 Cox proportional hazards regression models. Maximum leaf and bract sizes were

14 compared among plots by analysis of covariance (ANCOVA), in which initial bulb size

15 (2013) was included as a covariate after log-transformation. Tukey's HSD test was used

16 for post hoc multiple comparisons. Flower and fruit production per plant were analysed

17 using a GLM with a postulated Poisson error distribution with log-link function, in which

18 plot and initial bulb size in 2013 (after log-transformation) were set as explanatory

19 variables. Fruit-set success (fruit/flower ratio) and seed-set success (seed/ovule ratio)

20 were compared using a GLM with a postulated binomial error distribution, in which the

21 plot was set as an explanatory variable. Final bulb size (June 2014) was compared using a

22 GLM with a postulated gamma error distribution with log-link function, in which plot,

23 flower number and fruit number were set as explanatory variables and initial bulb size

24 (2013) was included as an offset variable after log-transformation. Pollen germination

25 rate and pollen tube length were compared using GLMs with a postulated binomial error

distribution with logit-link function and a gamma error distribution with log-link function, respectively, in which temperature (10, 15, 20, 25, 30°C) was set as an explanatory variable. All statistical analyses were conducted using an open source system, R version 3.0.1 (R Development Core Team 2013).

Results

Growth conditions and phenology

In the preliminary measurement of soil temperature in 2013, soil conditions were constantly maintained around 0–1°C during the winter (December to February) in every plot, indicating a lack of soil frost. Mean soil temperature during the growth period (April and May) was 7.3°C (ranging from 0.1°C to 20.2°C) in the forest plot. Daily mean soil temperature was 4.3ºC warmer in the GH plot and 0.6ºC warmer in the open plot in comparison with the forest plot (see Appendix Fig. 6).

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

1   Acceleration of snowmelt in the GH plot advanced both shoot growth initiation and

2   reproductive phenology (Fig. 1c). Although the duration of flowering period was similar

3   among plots (14, 15, and 17 days in the open, forest, and GH plot, respectively),

4   flowering started earlier in the GH plot (8 days after shoot emergence) compared with the

5   forest and open plots (16 days after shoot emergence).

6

7   Physiological traits of leaves and bracts

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9   Leaf P_{max} did not differ among plots ($P > 0.10$) but decreased with leaf age ($P < 0.001$;

10   Table 1a). A significant interaction existed between the GH plot and age ($P = 0.001$)

11   owing to a rapid decrease in P_{max} in the GH plot (Fig. 2a). This indicated that the period

12   of high photosynthesis was short in warm conditions. Leaf respiration rates tended to

13   decrease with leaf age in the open and forest plots but remained high in the GH plot,

14   especially at the fruiting stage, leading to a significant interaction between GH and age (P

15   < 0.001; Table 1c, Fig. 2c). Retention of high respiration rate during the fruiting period

16   indicated the high respiratory loss in the GH plot compared with the open and forest

17   conditions.

18   Bract P_{max} was slightly lower in the forest plot in comparison with the open and GH

19   plots, corresponding to lower irradiance under the canopy (Table 1b, Fig. 2b). Similar to

20   leaf P_{max}, bract P_{max} decreased with age ($P < 0.001$) and showed an interaction between

21   GH and age ($P < 0.001$) because of a rapid decrease in photosynthetic activity at fruiting

22   stage in warm conditions. Although bract respiration rates decreased with age in the open

23   and forest plots, plants in the GH plot retained high respiration rates at fruiting stage,

24   leading to a significant interaction between GH and age ($P = 0.006$; Table 1d, Fig. 2d).

25   Thus, warmer temperature caused greater respiratory loss also for bracts.

Leaf lifespan as well as bract lifespan varied significantly among plots (Fig. 3). Initiation of leaf senescence began 25, 34, and 34 days after shoot emergence in the GH, open, and forest plots, respectively. Mean ($\pm$ SE) leaf longevity was 43.3 ± 0.5, 49.4 ± 0.5, and 50.3 ± 0.4 days in the GH, open, and forest plots, respectively, whereas mean bract longevity was 42.1 ± 0.5, 48.8 ± 0.5, and 52.6 ± 0.4 days, respectively. Leaf longevity was significantly shortened in the GH plot ($P < 0.001$) but no difference between the open and forest plots ($P = 0.36$). Bract longevity was shortened in the open and GH plots compared with the forest plot, as shown by the Cox proportional hazard regression ($P < 0.001$, Fig. 3).

Maximum leaf and bract sizes increased with initial bulb size ($P = 0.015$ and $P < 0.001$, respectively) and varied among plots ($P < 0.001$; Table 2). Plants in the GH plot produced significantly smaller leaves (5.0 ± 0.3 cm^2) and bracts (1.7 ± 0.1 cm^2) compared with plants in the open plot (11.5 ± 0.5 cm^2 and 2.8 ± 0.2 cm^2, respectively) and the forest plot (11.2 ± 0.5 cm^2 and 2.7 ± 0.1 cm^2, respectively; $P < 0.05$ by Tukey's test).

Reproductive activity

Flower and fruit production in 2014 depended highly on bulb size in 2013 ($P < 0.001$; Appendix Table 5). There was no significant difference in flower number per plant among plots when size effect was considered using a GLM ($P > 0.05$; Table 3, Appendix Table 5). In contrast, fruit number per plant, fruit-set rate, and seed-set rate were significantly smaller in the GH plot ($P = 0.002$, $P = 0.001$ and $P < 0.001$, respectively), whereas they were similar between the forest and open plots ($P > 0.10$). These results indicated that reproductive success was restricted in the GH plot even when hand-pollination was conducted.

Bulb growth

Final bulb volume in June 2014 was significantly smaller in the GH plot ($P = 0.028$) but larger in the forest plot ($P < 0.001$) in comparison with the open plot (Table 4, Fig. 4). Flower production negatively influenced bulb size ($P = 0.048$), whereas fruit production did not ($P = 0.78$). Bulb size increased by 26% in the forest plot during the growth period, but showed a 14% decrease in the open plot and a 33% decrease in the GH plot. Therefore, vegetative growth was greatest in the original habitat under the canopy, but it was restricted in warm and constantly bright conditions.

Pollen germination activity

Mean pollen germination rates were 18.9%, 23.5%, 30.5%, 17.5%, and 7.9% at 10, 15, 20, 25, and 30ºC, respectively. The highest germination rate was recorded at the intermediate temperature (Fig. 5, Appendix Table 6). The highest temperature, which mimicked the GH conditions, significantly inhibited pollen germination ($P < 0.001$). Hence, high temperatures during the flowering period might have serious deleterious effects on fertilization success and subsequent seed production in *G. lutea*.

Mean values of pollen tube length were 0.88 ± 0.04 mm, 0.98 ± 0.04 mm, 1.08 ± 0.04 mm, 1.12 ± 0.04 mm, and 0.98 ± 0.05 mm at 10, 15, 20, 25, and 30ºC, respectively. The highest pollen tube growth was recorded at the intermediate temperature ($P < 0.001$; Appendix Table 6).

Discussion

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2   Responses of vegetative growth: leaf–bulb relationship

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4   Our experiment revealed that the physiological and phenological responses of leaves are

5   influenced more strongly by temperature than by light conditions, because their

6   photosynthetic activity, dark respiration rate, size, and longevity were similar between the

7   open and forest plots but clearly different in the GH plot. A rapid decrease in

8   photosynthetic activity, large respiration loss, small leaf area, and short lifespan in the

9   GH plot clearly indicated that the overall performance of assimilative ability was

10   negatively affected by warm conditions. These findings supported the previous studies on

11   other spring ephemeral plants (Lapointe and Lerat 2006; Badri et al. 2007; Lundmark et

12   al. 2009; Gandin et al. 2011).

13   Leaf P_{max} decreased quickly with time, reflecting the short lifespan of spring

14   ephemerals (Constable et al. 2007), but the decreasing rate was accelerated in the GH plot

15   (Fig. 2a), indicating earlier physiological aging in warm conditions. Dark respiration rates

16   in the GH plot stayed at a high level even during the fruiting period (Fig. 2c), suggesting

17   that most carbon fixed in this period might be exhausted by respiration loss. High

18   respiratory loss has been reported in other spring ephemerals grown in warm conditions

19   (Gardin et al. 2011; Bernatchez and Lapointe 2012). Shorter leaf longevity only in the

20   GH plot (Fig. 3a) indicated that leaf lifespan of spring ephemerals was more strongly

21   determined by temperature rather than light conditions. Yoshie (2008) reported an

22   extended longevity for *G. lutea* in cool growth temperatures in contrast to a summer-

23   green forb *Maianthemum dilatatum* in which cool temperatures shortened leaf lifespan.

24   Despite a relatively high P_{max} in the GH plot early in the season, reduced leaf longevity

25   and sizes, in addition to high respiration loss, should restrict carbon assimilation in warm

conditions, resulting in weaker source function. Especially, leaf size of plants in the GH

was less than half of plants in the open and forest plots. This might be caused by heat

stress in the GH, where daily maximum temperature often exceeded 30ºC (Fig. 1a).

Several studies have demonstrated that photosynthetic activity is regulated by the

sink intensity of storage organs (Sawada et al. 2003; Lundmark et al. 2009; Gaudin et al.

2011). In the spring geophyte *Erythronium americanum*, leaf senescence was induced by

a reduction in carbohydrate sink demand once the bulb was filled with carbohydrates

(Lapointe 2001). At a warm temperature, faster starch accumulation causes smaller sink

capacity of bulbs and leads to shorter leaf lifespan, resulting in smaller bulb size (Gandin

et al. 2011). A similar physiological mechanism is expected in *G. lutea*. Plants grown in

the forest plot developed the largest final bulb volume, whereas plants in the GH plot

accumulated least volume (Fig. 4a). Therefore, vegetative growth of *G. lutea* was

negatively influenced by warm condition as reported in other spring ephemerals such as *E.*

americanum (Lapointe 2001; Gandin et al. 2011), *Allium tricoccum* (Bernatchez and

Lapointe 2012), and *C. vernus* (Badri et al. 2007; Lundmark et al. 2009). On the other

hand, careful consideration is necessary to evaluate the climate change impacts on spring

ephemerals from this study, because warming effect by the GH was available only during

the daytime on clear days, while night-time temperatures were similar to the forest plot in

our experiment (Fig. 1a). Warm night conditions may accelerate the respiration loss of

plants, resulting in further decrease in bulb growth under warm conditions.

Bulb volume was also smaller in the open plot compared with the forest plot (Fig.

4b). This trend was in contrast to the previous report on this species (Sunmonu and Kudo

2014), where the bulb size was maintained when plants were transferred to an open

habitat during the second half of the growth season. Longer exposure to direct sunlight in

1   open conditions in the present experiment might have led to high transpiration and even

2   heat and water stress, resulting in the restriction of bulb growth.

3

4   Responses of reproductive characteristics: bract–fruit relationship

5

6   Onset of flowering occurred one week earlier from the initiation of growth in the GH plot

7   compared with the forest and open plots (Fig. 1c). This indicated that flowering initiation

8   was temperature dependent for plants growing in cool conditions (Fitter et al. 1995).

9   However, flowering duration and flower number were less affected by warming in our

10   experiment. The absence of changes in flowering period may be partly because hand-

11   pollination was performed soon after flowering for every flower. If floral longevity was

12   determined by the timing of pollen deposition on the stigmas (Primack 1985; Ishii and

13   Sakai 2000), this is a reasonable response. The previous warming experiment in a

14   subalpine meadow also documented earlier onset of flowering but no change in flowering

15   duration in warm conditions (Price and Waser 1998). The construction of aboveground

16   shoots in spring ephemeral herbs commonly depends on resources stored during the

17   preceding year, i.e., the size of storage organs (Muller 1978; Routhier and Lapointe 2002).

18   Flower production of *G. lutea* also depends on initial bulb size (Schrittler et al. 2009;

19   Sunmonu et al. 2013). Therefore, we predict that flower production in continuous warm

20   conditions may decrease from year to year in response to decreasing bulb size.

21   Although bract P_{max} in the forest plot was relatively small in comparison with the

22   open and GH plots during the early to middle growth period, the decline of P_{max}

23   progressed gradually toward fruiting stage in the forest plot, whereas P_{max} in the GH plot

24   was intensively suppressed at the fruiting stage (Fig. 2b). Similar to leaf responses, bracts

25   in the GH plot showed the shortest lifespan and smallest size, whereas longevity was

largest in the forest plot (Fig. 3b). Longer bract lifespan under the canopy may compensate for the smaller P_{max} of bracts in terms of assimilative function (Constable et al. 2007), resulting in similar fruit and seed production to the open plot. Short lifespan, rapid decrease in photosynthetic activity, high respiration rate even in the fruiting stage, and small size of bracts in the GH plot indicated low photosynthetic assimilation in warm conditions, as detected in the leaves. Because photosynthesis by bracts is completely responsible for seed production in this species (Sunmonu et al. 2013; Sunmonu and Kudo 2014), reduced bract assimilation in the GH plot might be responsible for the lower fruit and seed production.

Similar to the sink–source balance between bulb growth and leaf photosynthesis, as mentioned before, the sink intensity of fruits may also affect the photosynthetic activity and transportation of photosynthetic products to reproductive organs (Iglesias et al. 2002; Ida et al. 2013). When the sink intensity of the reproductive function decreased, the activity of photosynthetic function supporting the reproductive sink may be decreased, resulting in earlier senescence and shorter longevity (Iglesias et al. 2002). Low fertilization success causes the low sink intensity of developing fruits. The pollen germination rate of *G. lutea* was restricted at high temperature (Fig. 5), and it might have resulted in low fertilization success in the GH plot. Inhibitions of pollen vigour and stigma receptivity by heat stress were reported in some crop plants (Devasirvatham et al. 2012; Kaushal et al. 2013). If low reproductive output in the GH plot was caused by the failure of ovule fertilization, the small sink intensity of developing fruits might accelerate the senescence of bracts, which could lead to feedback restriction of fruit development. Our previous study revealed that photosynthetic products from bracts were flexibly used for bulb growth when all flowers were removed (Sunmonu and Kudo 2014). Because bulb growth seemed to be stopped at the fruiting stage in the GH plot, the small sink

1 intensity of both bulb and fruits might accelerate the senescence of bracts at the same

2 time as leaf senescence.

3   Contrary to our previous study (Sunmonu and Kudo 2014), seed-set rates did not

4 differ between the open and forest plots (Table 3), indicating no advantage of an extended

5 bright period for seed production in this year. A previous study reported higher seed-set

6 success in plants inhabiting a forest-edge habitat compared with plants growing beneath a

7 forest canopy (Nishikawa 2009). These contradictory results indicate that the advantage

8 of longer bright conditions may vary from year to year. Growth initiation of *G. lutea* in

9 natural conditions in 2014 occurred one week earlier than an average year owing to

10 earlier snowmelt (Fig. 1c). Thus, most reproductive plants might have completed seed

11 production by the time of canopy closure, thereby making light-resource limitation for

12 seed production undetectable in an early-snowmelt year.

13

14   In conclusion, cool temperature early in the spring in a forest is beneficial for spring

15 ephemerals, and future climate warming is predicted to be detrimental to the growth and

16 reproduction of spring ephemerals. On the other hand, the effects of early canopy closure

17 on the fitness of spring ephemerals are not consistent depending on whether the bright

18 period from snowmelt to canopy closure was longer or shorter than the period for the

19 completion of bulb growth and seed production. Reproductive mode, i.e., the balance

20 between sexual reproduction and vegetative reproduction, is crucial to predict the fate of

21 population dynamics of perennial herbs under conditions of global climate change. *G.*

22 *lutea* can reproduce by both seed production (sexual reproduction) at large size and

23 bulbils (vegetative reproduction) at small size (Schnittler et al. 2009). Because we

24 focused on the responses of only reproductive plants with a large size in the present study,

25 it is unclear how climate warming affects bulbile production in this species. Therefore,

long-term monitoring of the 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.

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21   a spring ephemeral (*Gagea lutea)* and a summergreen forb (*Maianthemum dilatatum).*

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23

24

Figure legends

3   **Fig. 1.** Fluctuations in (a) air temperature and (b) photosynthetically active radiation

4   (PAR) during the growth season of *Gagea lutea* in each plot. PAR was measured only in

5   the forest and open plots. (c) Green leaf period and flowering period in each plot in 2014.

6   **Fig. 2.** Seasonal transitions in leaf P_{max} (a), bract P_{max} (b), leaf dark respiration rate (c),

7   and bract dark respiration rate (d) in the open, forest, and greenhouse (GH) plots. The age

8   of plants is expressed as days since shoot emergence. Measurements were conducted at

9   pre-flowering, flowering, and fruit developing stages in each plot. Mean ± SE. See Table

10 1 for statistical results.

11 **Fig. 3.** Survival curves of leaves (a) and bracts (b) in the open, forest, and greenhouse

12 (GH) plots in 2014. Results of a generalized linear model are indicated (*** $P < 0.001$).

13 **Fig. 4.** (a) Final bulb size in volume (cm^3) and (b) change in bulb volume during a growth

14 period in the open, forest, and greenhouse (GH) plots. Results of a generalized linear

15 model are indicated (* $P < 0.05$, *** $P < 0.0001$). Box plots indicate 25, 50 and 75

16 percentiles, and whiskers indicate 10 and 90 percentiles of the data distribution. See

17 Table 4 for details for the statistical results.

18 **Fig. 5.** Pollen germination rates under various temperatures. Results of a generalized

19 linear model are indicated (ns $P > 0.05$, * $P < 0.05$, *** $P < 0.0001$) in which 10ºC is set

20 as an interception. Box plots indicate 25, 50 and 75 percentiles, and whiskers indicate 10

21 and 90 percentiles of the data distribution. See Appendix Table 6 for details of the

22 statistical results.

23 **Appendix**

24 **Fig. 6.** Soil temperature during the growth period in 2013 in each plot. Measurements

25 were performed at the depth of 10 cm at hourly intervals.

26

Table 1. Results of generalized linear models for P_{max} and dark respiration of leaves and bracts among plots (open, forest, greenhouse [GH]) and shoot age

Variable	Coefficient	SE	t value	P value
(a) Leaf P_{max}				
Intercept (Open)	3.67	0.18	20.31	<0.001
Forest	-0.22	0.26	-0.86	0.39
GH	0.31	0.24	1.30	0.20
Age	-0.32	0.01	-5.85	<0.001
Forest ×Age	-0.005	0.008	0.67	0.51
GH ×Age	0.028	0.008	-3.44	0.001
(b) Bract P_{max}				
Intercept (Open)	3.13	0.18	17.34	<0.001
Forest	-0.54	0.26	-2.09	0.043
GH	1.08	0.27	4.07	<0.001
Age	-0.020	0.005	-3.67	<0.001
Forest ×Age	0.011	0.008	1.45	0.15
GH ×Age	-0.063	0.010	-6.17	<0.001
(c) Leaf respiration				
Intercept (Open)	1.30	0.13	9.76	<0.001
Forest	0.009	0.19	0.05	0.96
GH	-0.47	0.17	-2.73	0.009
Age	-0.027	0.004	-6.67	<0.001
Forest ×Age	0.008	0.006	1.32	0.19
GH ×Age	0.029	0.006	4.68	<0.001
(d) Bract respiration				
Intercept (Open)	0.98	0.24	4.09	<0.001
Forest	-0.15	0.34	-0.43	0.67
GH	-0.58	0.35	-1.65	0.11
Age	-0.023	0.007	-3.15	0.003
Forest ×Age	0.011	0.010	1.08	0.29
GH ×Age	0.040	0.014	2.93	0.006

1

Table 2. Results of ANCOVA for maximum leaf and bract size among plots (open, forest, greenhouse [GH]) according to bulb size (2013) as representative of plant size

Variables	SS	df	F	P value
(a) Leaf size				
Intercept	5790	1	656.7	<0.001
Plot	1415	2	80.2	<0.001
log (bulb size)	53	1	6.0	0.015
Residuals	1279	145		
(b) Bract size				
Intercept	355	1	362.3	<0.001
Plot	53	2	26.9	<0.001
log (bulb size)	21	1	21.4	<0.001
Residuals	142	145		

2

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28

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Table 3. Flower, fruit and seed production in the forest, open, and greenhouse [GH] plots. Mean ± SE

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

** $P < 0.01$, *** $P < 0.001$ by generalized linear model. See Appendix Table 5 for statistical results.

2

3

1

Table 4. Result of generalized linear model for final bulb size (2014), in which plot (open, forest, greenhouse [GH]), flower production, and fruit production were set as explanatory variables

Variable	Coefficient	SE	t value	P value
Intercept (Open)	0.202	0.121	1.62	0.097
Forest	0.314	0.083	3.78	<0.001
GH	-0.186	0.084	-2.22	0.028
Flower No.	-0.075	0.038	-2.00	0.048
Fruit No.	0.011	0.037	0.28	0.775

2

3

Appendix

Table 5. Results of generalized linear models for reproductive performance in open, forest and greenhouse [GH] plots

Variables	Coefficient	SE	*Z* value	*P* value
(a) Flower production				
Intercept (Open)	1.53	0.06	24.25	< 0.001
Forest	-0.17	0.10	-1.72	0.085
GH	-0.14	0.09	-1.51	0.132
log (bulb size)	0.52	0.13	4.16	< 0.001
(b) Fruit production				
Intercept (Open)	1.38	0.07	18.98	
Forest	-0.16	0.11	-1.45	0.14
GH	-0.35	0.11	-3.08	0.002
log (bulb size)	0.52	0.15	3.49	< 0.001
(c) Fruit set				
Intercept (Open)	1.71	0.18	9.59	< 0.001
Forest	0.23	0.29	0.79	0.43
GH	-0.80	0.24	-3.28	0.001
(d) Seed set				
Intercept (Open)	0.80	0.03	26.91	< 0.001
Forest	-0.05	0.04	-1.03	0.31
GH	-0.23	0.05	-5.06	< 0.001

(a)

Air temperature (°C)

- Forest
- Open
- GH

April   May

(b)

Daily PAR (μmol m⁻² s⁻¹)

- Forest
- Open

(c)

GH

Forest

Open

Flowering period

Leafing period

Julian date

Fig. 1 (Sunmonu & Kudo)

Fig. 2 (Sunmonu & Kudo)

(a)

Leaf survuval rate

Open, Forest > GH***

Forest
Open
GH

(b)

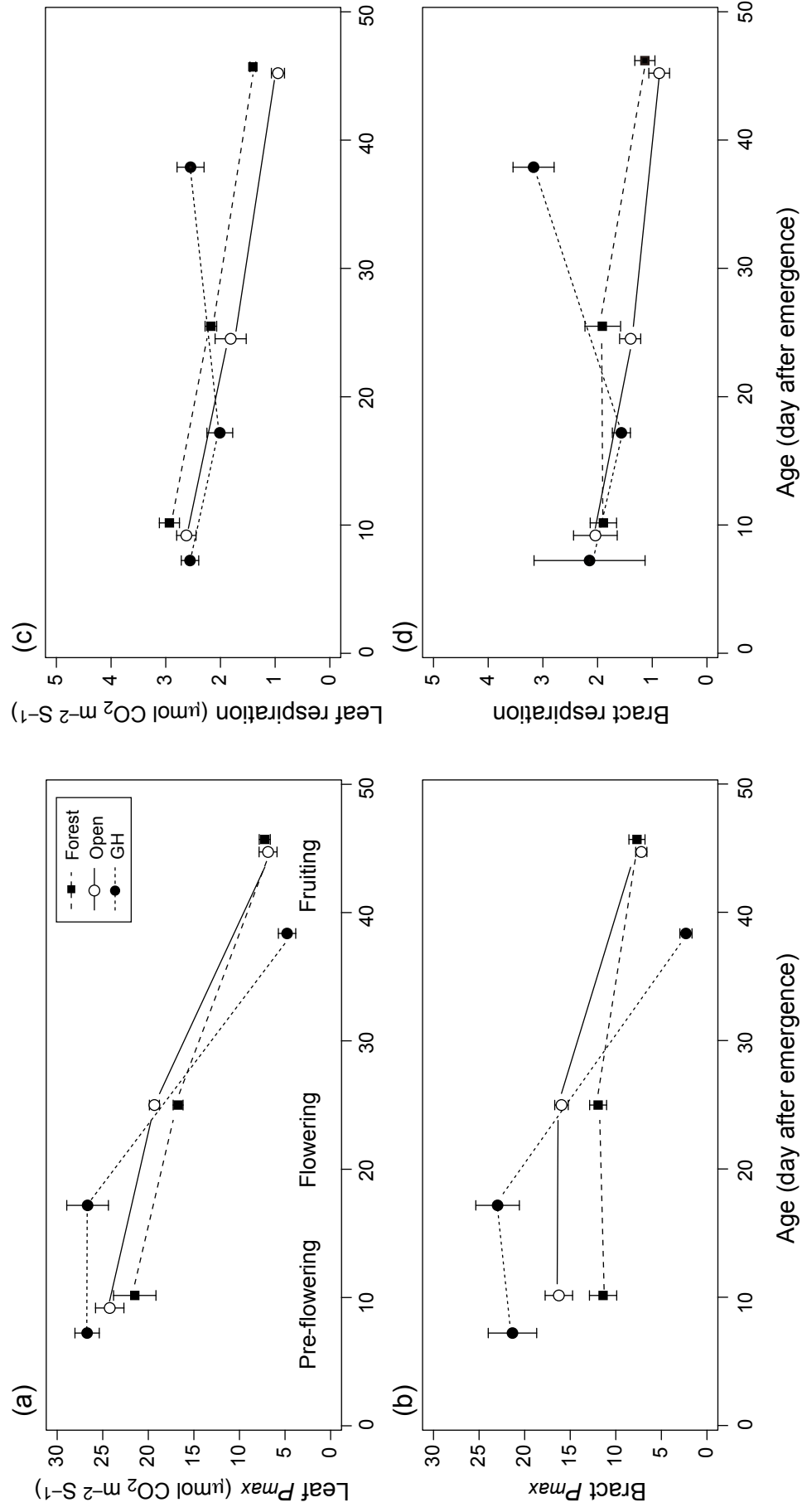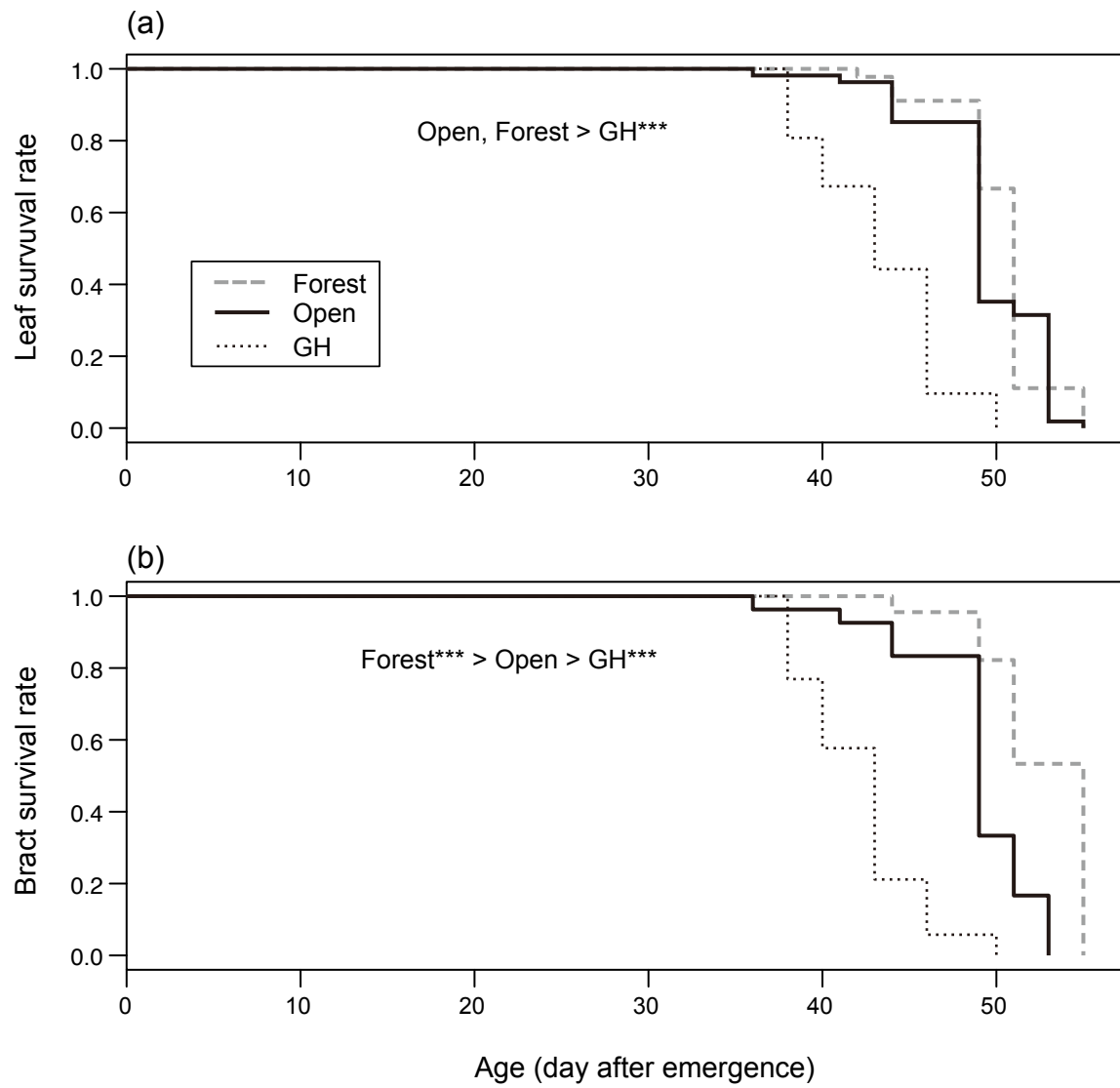Bract survival rate

Forest*** > Open > GH***
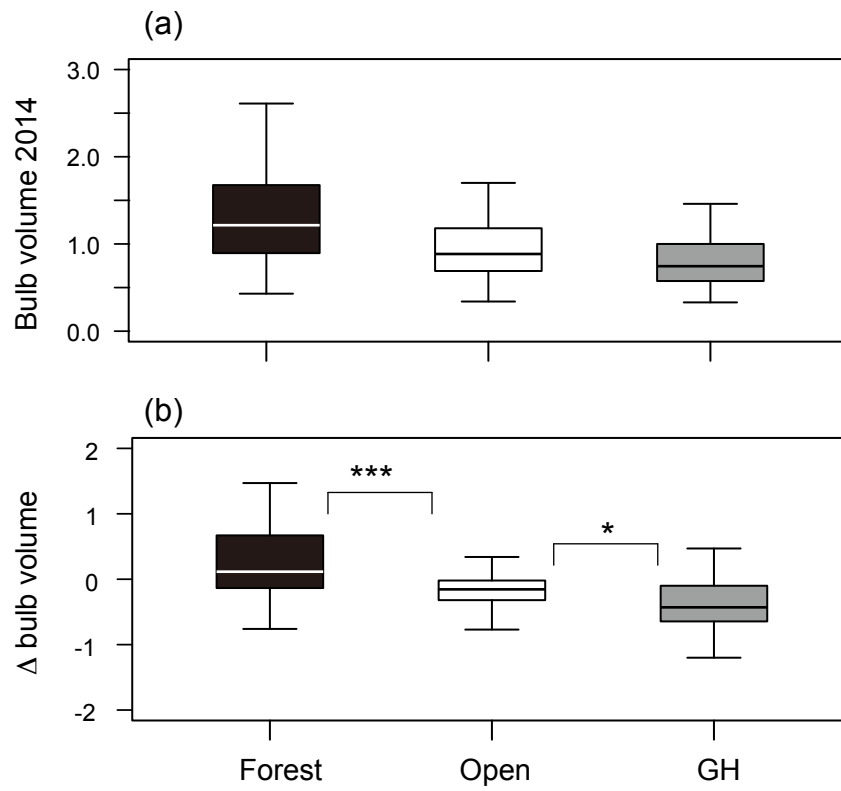
Age (day after emergence)
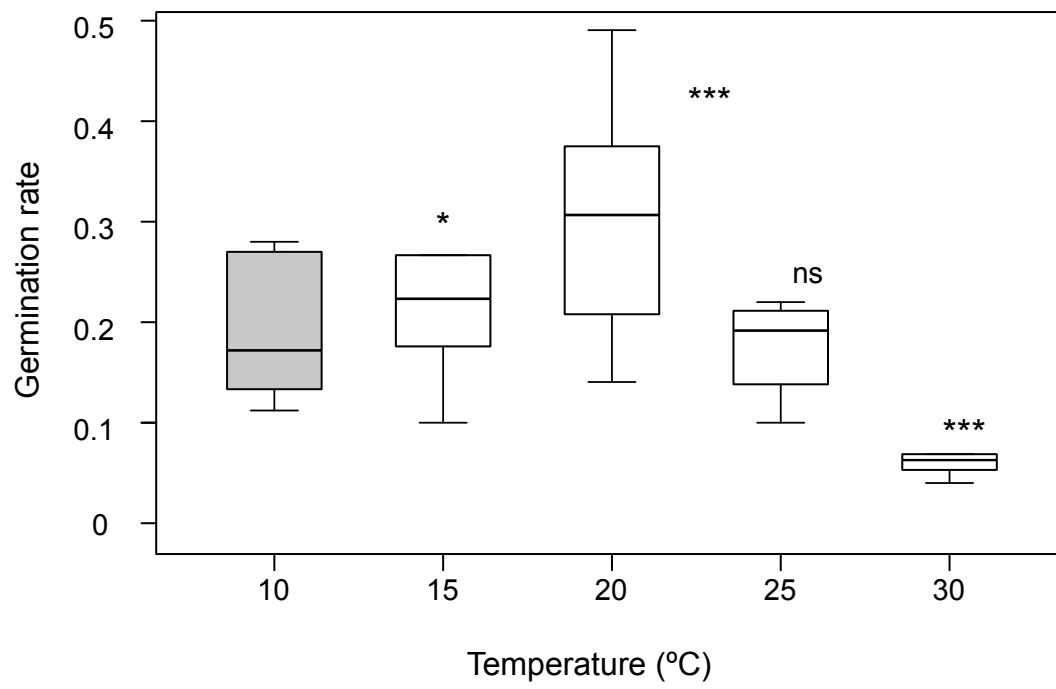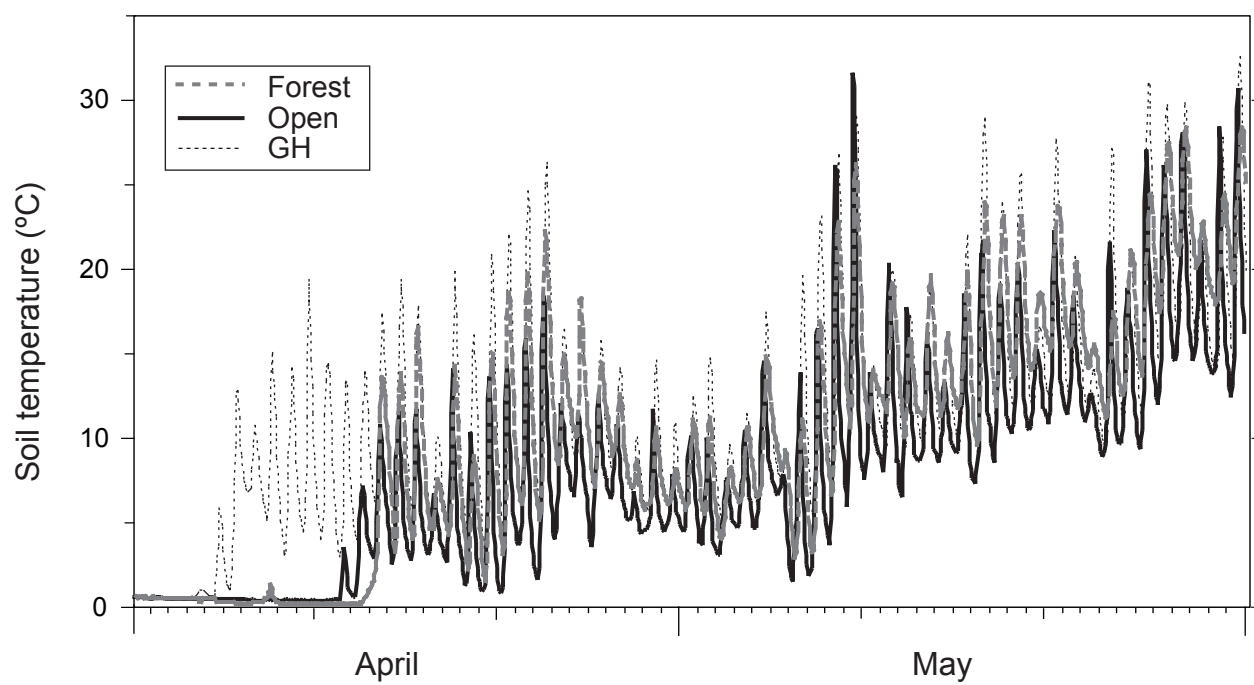
Fig. 3 (Sunmonu & Kudo)

Fig. 4 (Sunmonu & Kudo)

Fig. 5 (Sunmonu & Kudo)

Appendix Fig. 6 (Sunmonu & Kudo)