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3 Warm temperature conditions restrict the sexual reproduction and vegetative  
4 growth of the spring ephemeral *Gagea lutea* (Liliaceae)

5

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11

12 Running title: Effect of warming on spring herbs

13

1 **Abstract**

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

22

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

25

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

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

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

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

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

3 In this study, we explored the hypothesis that the extent of reproductive output and  
4 bulb growth in warm conditions would depend on the responses to source organs for each  
5 sink function (i.e., bract for seed production and leaf for bulb growth). Seed production  
6 and bulb growth may not be sensitive to climate change if the lifespan and carbon  
7 assimilation of the leaf and bract are not restricted under warm conditions. Apart from the  
8 source–sink balance for resource allocation, warmer temperature may directly influence  
9 the pre-zygote process even under conditions of good pollination success, i.e.,  
10 fertilization success, such as pollen viability, pollen tube growth, stigma receptivity, and  
11 ovule viability, which may also decrease seed production (Hedhly et al. 2008). Because  
12 pollen activity is generally sensitive to temperature (Hedhly et al. 2005; Kakani et al.  
13 2005), the thermal influence on pollen performance may also influence reproductive  
14 success in a warm climate.

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

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

3

#### 4 **Materials and methods**

5

##### 6 Study species and experimental design

7

8 *Gagea lutea* Ker-Gawl. (Liliaceae) is a polycarpic perennial herbaceous species  
9 inhabiting northern temperate forests. This species has a typical spring ephemeral  
10 lifecycle; flowering starts immediately after snowmelt concurrently with leaf expansion  
11 (in mid- to late April) and fruits mature about two weeks after anthesis. It produces 1–10  
12 flowers and 24–39 ovules per plant on average (Nishikawa, 1998). It is pollinated by  
13 insects, and cross-pollination is more effective for seed production (Kudo *et al.*, 2004).  
14 Aboveground shoots usually die at the same time as seed dispersal, at the time of canopy  
15 closure in late May. Thus, the short period between snowmelt and canopy closure is when  
16 this species accumulates resources in the underground bulb. Non-reproductive individuals  
17 produce only one leaf, while one basal leaf and a pair of long and short leaf-like bracts on  
18 the scape are produced in reproductive plants (Sunmonu *et al.*, 2013).

19 Bulbs of *G. lutea* were collected (n = 203) from a secondary deciduous forest within  
20 the campus of Hokkaido University, Sapporo, Japan (43° 04' 57" N, 141° 20' 22" E) in late  
21 autumn of 2012. This forest is usually covered with snow from early December to early  
22 April, and common canopy trees in this fragmented forest include *Ulmus davidiana* var.  
23 *japonica*, *Cercidiphyllum japonica*, *Betula platyphylla* var. *japonica* and *Populus*  
24 *maximowiczii*. The bulbs were immediately taken to the laboratory and their volumes  
25 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

22

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

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

9

#### 10 Physiological measurements of leaves and bracts

11

12 After shoot emergence in spring 2014, all reproductive plants producing floral buds were  
13 monitored. To investigate the physiological responses of leaves and bracts to  
14 environmental manipulations among plots, leaf and bract maximum photosynthetic rates  
15 ( $P_{max}$ ) at saturation irradiance ( $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and dark respiration rates were  
16 measured using a portable LI-6400 photosynthesis system (Li-Cor, Lincoln, NE, USA).  
17 Three of the experimental plants were selected per plot at each of three growth stages: 7th  
18 April at floral-bud stage, 16th April at flowering stage, and 8th May at early fruiting stage  
19 in the GH plot; 18th April, 12th May, and 23rd May in the forest plot; and 18th April,  
20 10th May, and 24th May in the open plot. Respiration rate was measured after leaving the  
21 leaf for 5–7 min in the dark ( $0 \mu\text{mol m}^{-2} \text{s}^{-1}$  irradiance). Leaf temperature in the chamber  
22 was controlled at  $20^{\circ}\text{C}$ , and the concentration of  $\text{CO}_2$  in ambient air entering the leaf  
23 chamber was maintained at  $380 \mu\text{mol mol}^{-1}$ . This temperature corresponded to the typical  
24 daytime temperature on clear days in the growth period of *G. lutea* (see results) in which  
25 photosynthetic activity is maximal (Sunmonu et al. 2013). Leaf-to-air vapor pressure

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

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

14

#### 15 Reproductive output

16

17 To evaluate the reproductive output in various environmental conditions, the number of  
18 floral buds was recorded for each plant in every plot during the flowering period. Then,  
19 artificial outcrossing by hand pollination was conducted for every flower to eliminate  
20 pollen limitation for seed production. Soon before seed dispersal, all infructescences were  
21 harvested and taken to the laboratory, where individual fruits were opened carefully and  
22 the numbers of mature seeds and undeveloped ovules in each fruit were counted. Fruit-set  
23 ratio was expressed as matured fruit number divided by original flower number, and seed  
24 production was taken as the ratio of mature seed number to original ovule number

1 produced per plant. Duration of the flowering period was also recorded for all plots to  
2 clarify any environmental effects on flowering phenology.

3

#### 4 Bulb growth

5

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

13

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

8

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

1 distribution with logit-link function and a gamma error distribution with log-link function,  
2 respectively, in which temperature (10, 15, 20, 25, 30°C) was set as an explanatory  
3 variable. All statistical analyses were conducted using an open source system, R version  
4 3.0.1 (R Development Core Team 2013).

5

## 6 **Results**

7

### 8 Growth conditions and phenology

9

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

16 Air temperature during the experimental period in 2014 showed a similar trend to  
17 soil temperature in 2013. Mean air temperature throughout the growth period (April and  
18 May) was 10.2°C (ranging from -1.2°C to 28.0°C) in the forest plot. Daily mean  
19 temperatures in the GH and open plots were 3.5°C and 0.8°C, respectively, warmer than  
20 the forest plot (Fig. 1a). Daily maximum temperatures in the GH and open plots were  
21 15.3°C and 3.1°C, respectively, warmer than in the forest plot. PAR in the open plot was  
22 two times larger than that in the forest plot (Fig. 1b). As the season progressed, the  
23 difference in PAR between the open and forest habitats became larger owing to  
24 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

8  
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.

1 Leaf lifespan as well as bract lifespan varied significantly among plots (Fig. 3).  
2 Initiation of leaf senescence began 25, 34, and 34 days after shoot emergence in the GH,  
3 open, and forest plots, respectively. Mean ( $\pm$  SE) leaf longevity was  $43.3 \pm 0.5$ ,  $49.4 \pm 0.5$ ,  
4 and  $50.3 \pm 0.4$  days in the GH, open, and forest plots, respectively, whereas mean bract  
5 longevity was  $42.1 \pm 0.5$ ,  $48.8 \pm 0.5$ , and  $52.6 \pm 0.4$  days, respectively. Leaf longevity  
6 was significantly shortened in the GH plot ( $P < 0.001$ ) but no difference between the  
7 open and forest plots ( $P = 0.36$ ). Bract longevity was shortened in the open and GH plots  
8 compared with the forest plot, as shown by the Cox proportional hazard regression ( $P <$   
9  $0.001$ , Fig. 3).

10 Maximum leaf and bract sizes increased with initial bulb size ( $P = 0.015$  and  $P <$   
11  $0.001$ , respectively) and varied among plots ( $P < 0.001$ ; Table 2). Plants in the GH plot  
12 produced significantly smaller leaves ( $5.0 \pm 0.3 \text{ cm}^2$ ) and bracts ( $1.7 \pm 0.1 \text{ cm}^2$ ) compared  
13 with plants in the open plot ( $11.5 \pm 0.5 \text{ cm}^2$  and  $2.8 \pm 0.2 \text{ cm}^2$ , respectively) and the forest  
14 plot ( $11.2 \pm 0.5 \text{ cm}^2$  and  $2.7 \pm 0.1 \text{ cm}^2$ , respectively;  $P < 0.05$  by Tukey's test).

15

## 16 Reproductive activity

17

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

1

## 2 Bulb growth

3

4 Final bulb volume in June 2014 was significantly smaller in the GH plot ( $P = 0.028$ ) but  
5 larger in the forest plot ( $P < 0.001$ ) in comparison with the open plot (Table 4, Fig. 4).

6 Flower production negatively influenced bulb size ( $P = 0.048$ ), whereas fruit production  
7 did not ( $P = 0.78$ ). Bulb size increased by 26% in the forest plot during the growth period,  
8 but showed a 14% decrease in the open plot and a 33% decrease in the GH plot.

9 Therefore, vegetative growth was greatest in the original habitat under the canopy, but it  
10 was restricted in warm and constantly bright conditions.

11

## 12 Pollen germination activity

13

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

20 Mean values of pollen tube length were  $0.88 \pm 0.04$  mm,  $0.98 \pm 0.04$  mm,  $1.08 \pm$   
21  $0.04$  mm,  $1.12 \pm 0.04$  mm, and  $0.98 \pm 0.05$  mm at 10, 15, 20, 25, and 30°C, respectively.

22 The highest pollen tube growth was recorded at the intermediate temperature ( $P < 0.001$ ;  
23 Appendix Table 6).

24

## 25 Discussion

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Responses of vegetative growth: leaf–bulb relationship

Our experiment revealed that the physiological and phenological responses of leaves are influenced more strongly by temperature than by light conditions, because their photosynthetic activity, dark respiration rate, size, and longevity were similar between the open and forest plots but clearly different in the GH plot. A rapid decrease in photosynthetic activity, large respiration loss, small leaf area, and short lifespan in the GH plot clearly indicated that the overall performance of assimilative ability was negatively affected by warm conditions. These findings supported the previous studies on other spring ephemeral plants (Lapointe and Lerat 2006; Badri et al. 2007; Lundmark et al. 2009; Gardin et al. 2011).

Leaf  $P_{max}$  decreased quickly with time, reflecting the short lifespan of spring ephemerals (Constable et al. 2007), but the decreasing rate was accelerated in the GH plot (Fig. 2a), indicating earlier physiological aging in warm conditions. Dark respiration rates in the GH plot stayed at a high level even during the fruiting period (Fig. 2c), suggesting that most carbon fixed in this period might be exhausted by respiration loss. High respiratory loss has been reported in other spring ephemerals grown in warm conditions (Gardin et al. 2011; Bernatchez and Lapointe 2012). Shorter leaf longevity only in the GH plot (Fig. 3a) indicated that leaf lifespan of spring ephemerals was more strongly determined by temperature rather than light conditions. Yoshie (2008) reported an extended longevity for *G. lutea* in cool growth temperatures in contrast to a summer-green forb *Maianthemum dilatatum* in which cool temperatures shortened leaf lifespan. Despite a relatively high  $P_{max}$  in the GH plot early in the season, reduced leaf longevity and sizes, in addition to high respiration loss, should restrict carbon assimilation in warm

1 conditions, resulting in weaker source function. Especially, leaf size of plants in the GH  
2 was less than half of plants in the open and forest plots. This might be caused by heat  
3 stress in the GH, where daily maximum temperature often exceeded 30°C (Fig. 1a).

4 Several studies have demonstrated that photosynthetic activity is regulated by the  
5 sink intensity of storage organs (Sawada et al. 2003; Lundmark et al. 2009; Gaudin et al.  
6 2011). In the spring geophyte *Erythronium americanum*, leaf senescence was induced by  
7 a reduction in carbohydrate sink demand once the bulb was filled with carbohydrates  
8 (Lapointe 2001). At a warm temperature, faster starch accumulation causes smaller sink  
9 capacity of bulbs and leads to shorter leaf lifespan, resulting in smaller bulb size (Gandin  
10 et al. 2011). A similar physiological mechanism is expected in *G. lutea*. Plants grown in  
11 the forest plot developed the largest final bulb volume, whereas plants in the GH plot  
12 accumulated least volume (Fig. 4a). Therefore, vegetative growth of *G. lutea* was  
13 negatively influenced by warm condition as reported in other spring ephemerals such as *E.*  
14 *americanum* (Lapointe 2001; Gandin et al. 2011), *Allium tricoccum* (Bernatchez and  
15 Lapointe 2012), and *C. vernus* (Badri et al. 2007; Lundmark et al. 2009). On the other  
16 hand, careful consideration is necessary to evaluate the climate change impacts on spring  
17 ephemerals from this study, because warming effect by the GH was available only during  
18 the daytime on clear days, while night-time temperatures were similar to the forest plot in  
19 our experiment (Fig. 1a). Warm night conditions may accelerate the respiration loss of  
20 plants, resulting in further decrease in bulb growth under warm conditions.

21 Bulb volume was also smaller in the open plot compared with the forest plot (Fig.  
22 4b). This trend was in contrast to the previous report on this species (Sunmonu and Kudo  
23 2014), where the bulb size was maintained when plants were transferred to an open  
24 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

1 largest in the forest plot (Fig. 3b). Longer bract lifespan under the canopy may  
2 compensate for the smaller  $P_{max}$  of bracts in terms of assimilative function (Constable et  
3 al. 2007), resulting in similar fruit and seed production to the open plot. Short lifespan,  
4 rapid decrease in photosynthetic activity, high respiration rate even in the fruiting stage,  
5 and small size of bracts in the GH plot indicated low photosynthetic assimilation in warm  
6 conditions, as detected in the leaves. Because photosynthesis by bracts is completely  
7 responsible for seed production in this species (Sunmonu et al. 2013; Sunmonu and Kudo  
8 2014), reduced bract assimilation in the GH plot might be responsible for the lower fruit  
9 and seed production.

10         Similar to the sink–source balance between bulb growth and leaf photosynthesis, as  
11 mentioned before, the sink intensity of fruits may also affect the photosynthetic activity  
12 and transportation of photosynthetic products to reproductive organs (Iglesias et al. 2002;  
13 Ida et al. 2013). When the sink intensity of the reproductive function decreased, the  
14 activity of photosynthetic function supporting the reproductive sink may be decreased,  
15 resulting in earlier senescence and shorter longevity (Iglesias et al. 2002). Low  
16 fertilization success causes the low sink intensity of developing fruits. The pollen  
17 germination rate of *G. lutea* was restricted at high temperature (Fig. 5), and it might have  
18 resulted in low fertilization success in the GH plot. Inhibitions of pollen vigour and  
19 stigma receptivity by heat stress were reported in some crop plants (Devasirvatham et al.  
20 2012; Kaushal et al. 2013). If low reproductive output in the GH plot was caused by the  
21 failure of ovule fertilization, the small sink intensity of developing fruits might accelerate  
22 the senescence of bracts, which could lead to feedback restriction of fruit development.  
23 Our previous study revealed that photosynthetic products from bracts were flexibly used  
24 for bulb growth when all flowers were removed (Sunmonu and Kudo 2014). Because  
25 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,

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

4

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6

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10

11

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21 a spring ephemeral (*Gagea lutea*) and a summergreen forb (*Maianthemum dilatatum*).  
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- 23
- 24

1 **Figure legends**

2

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  $\pm$  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 ( $\text{cm}^3$ ) 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  $10^\circ\text{C}$  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	<i>t</i> value	<i>P</i> value
<b>(a) Leaf <math>P_{max}</math></b>				
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>(b) Bract <math>P_{max}</math></b>				
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
<b>(c) Leaf respiration</b>				
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
<b>(d) Bract respiration</b>				
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	<i>F</i>	<i>P</i> value
<b>(a) Leaf size</b>				
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>(b) Bract size</b>				
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

3

1

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

Plot	N	Flower no.	Fruit no.	Fruit set	Seed set
Forest	45	3.9 $\pm$ 0.2	3.4 $\pm$ 0.2	0.89 $\pm$ 0.03	0.59 $\pm$ 0.03
Open	49	4.9 $\pm$ 0.2	4.2 $\pm$ 0.3	0.83 $\pm$ 0.02	0.58 $\pm$ 0.02
GH	40	4.5 $\pm$ 0.2	3.2 $\pm$ 0.2**	0.73 $\pm$ 0.04***	0.47 $\pm$ 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	<i>t</i> value	<i>P</i> 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
<b>(a) Flower production</b>				
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>(b) Fruit production</b>				
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
<b>(c) Fruit set</b>				
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
<b>(d) Seed set</b>				
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

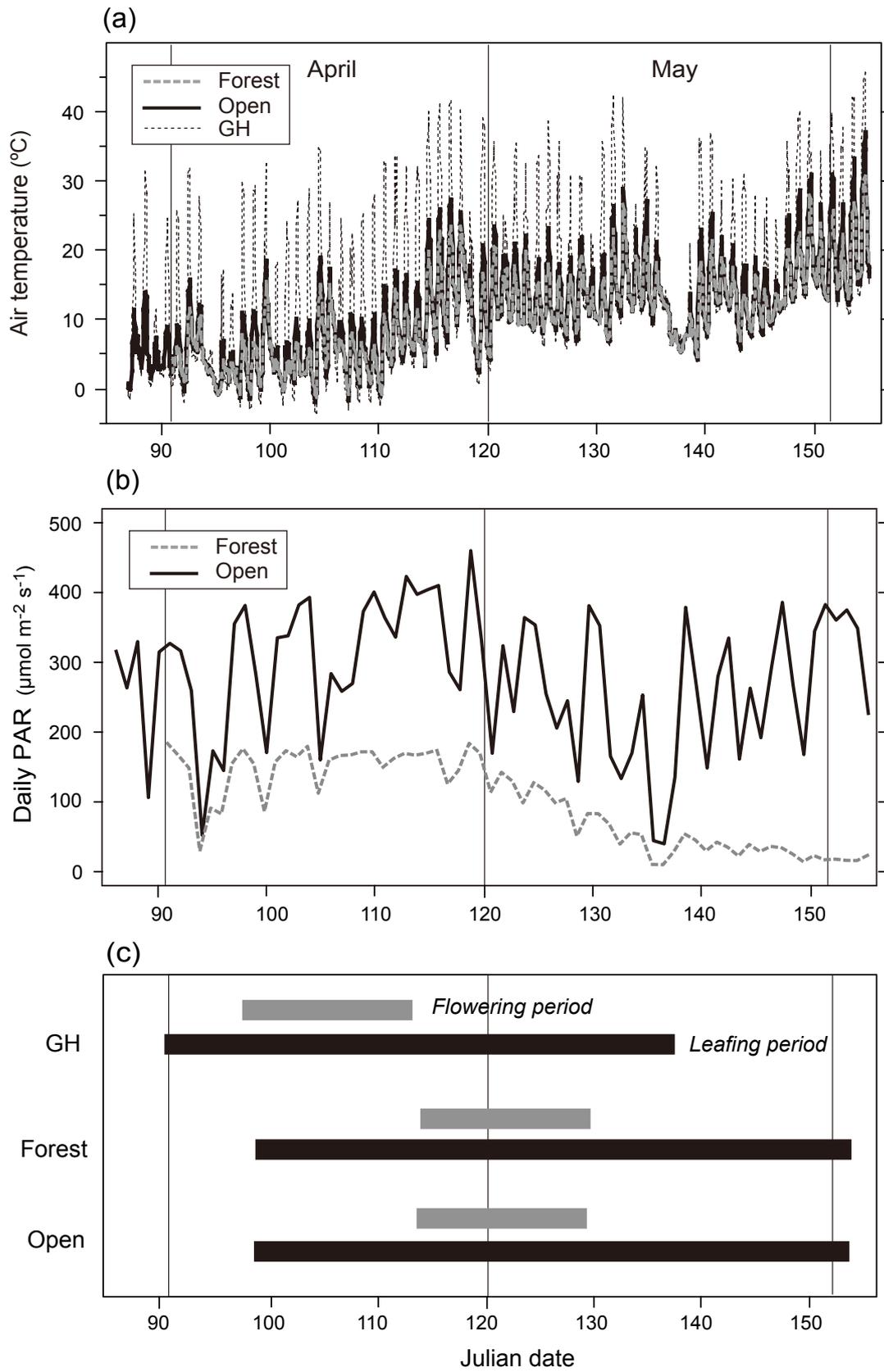


Fig. 1 (Sunmonu & Kudo)

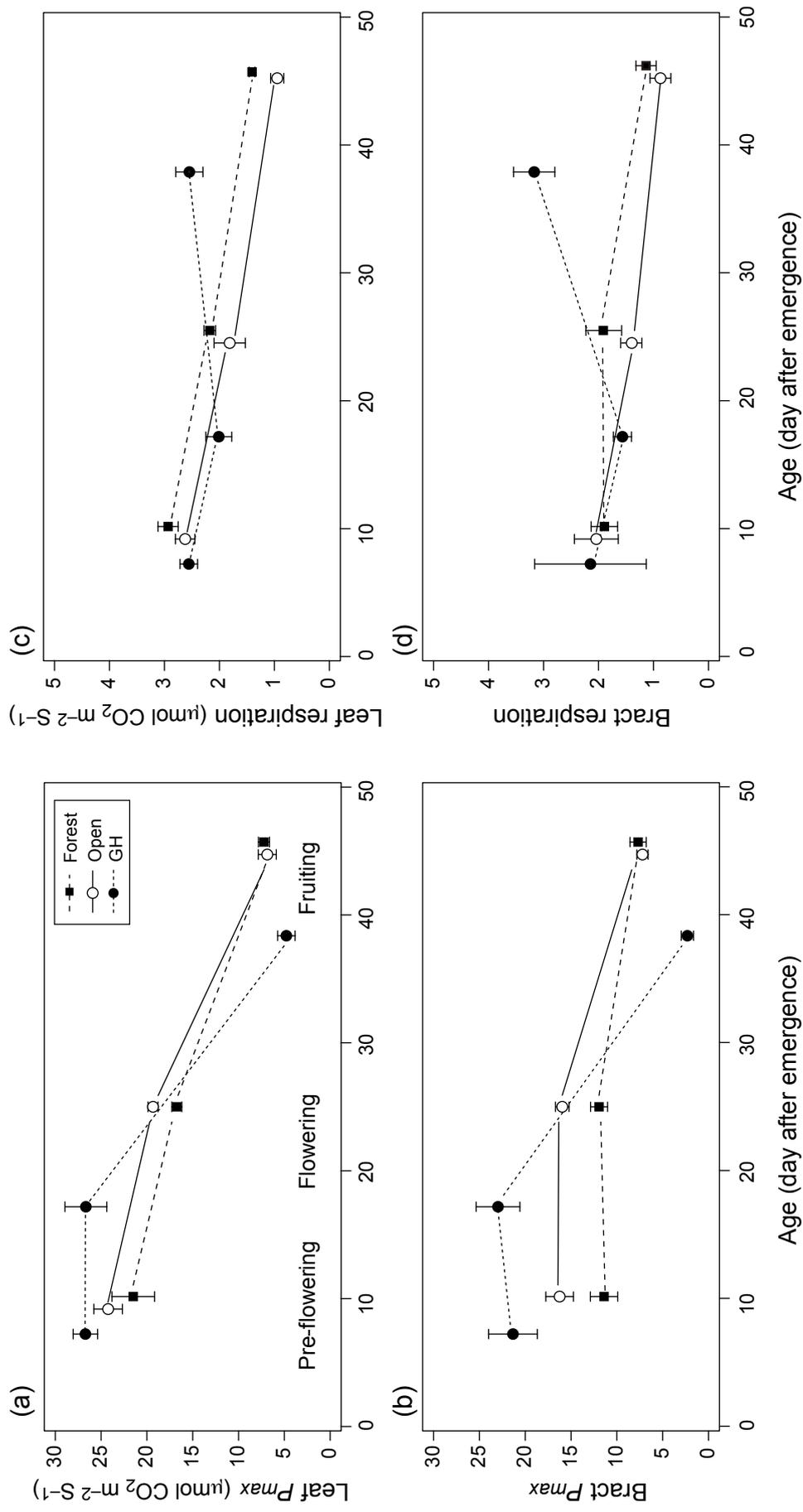


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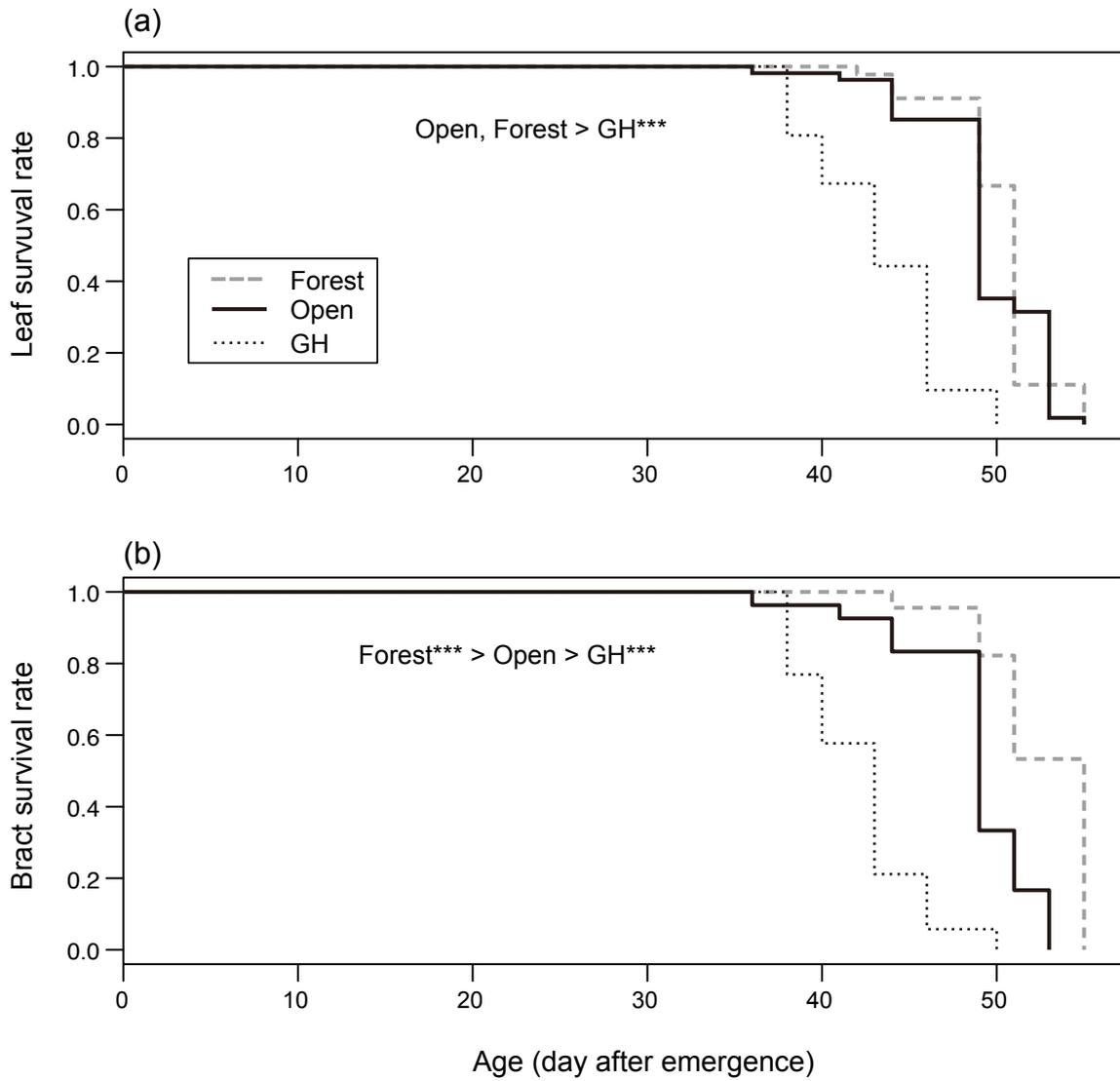


Fig. 3 (Sunmonu & Kudo)

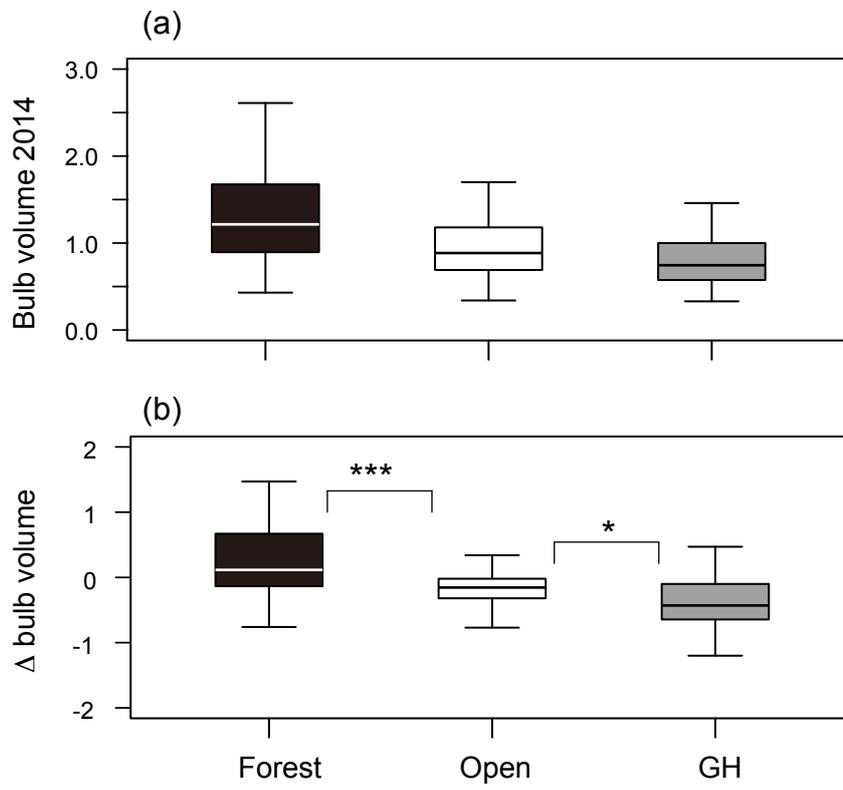


Fig. 4 (Sunmonu & Kudo)

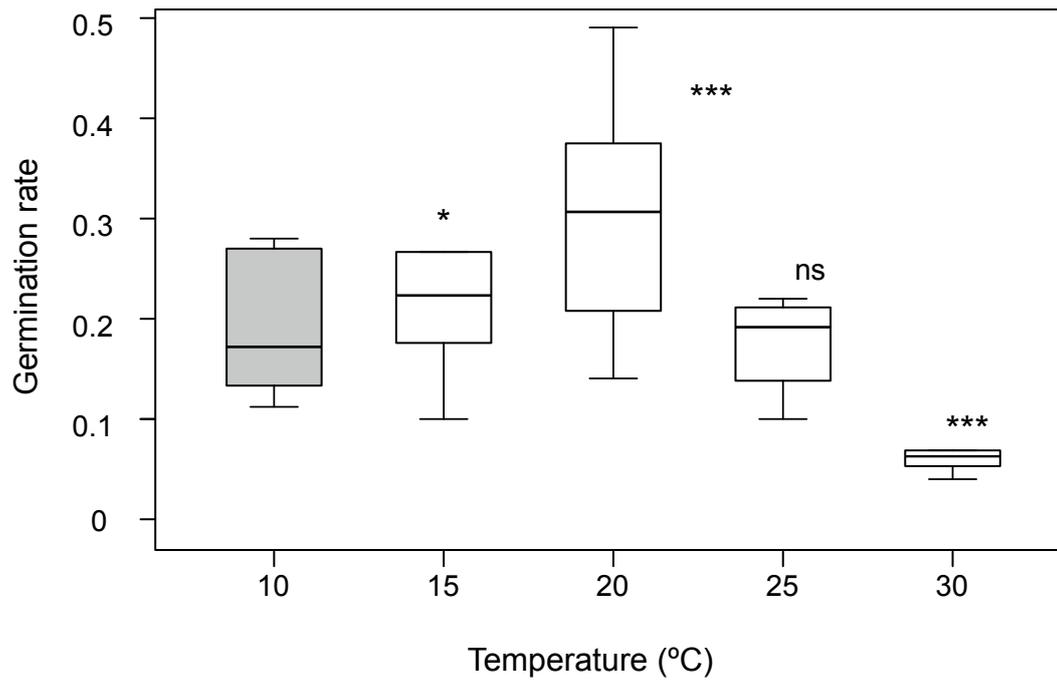
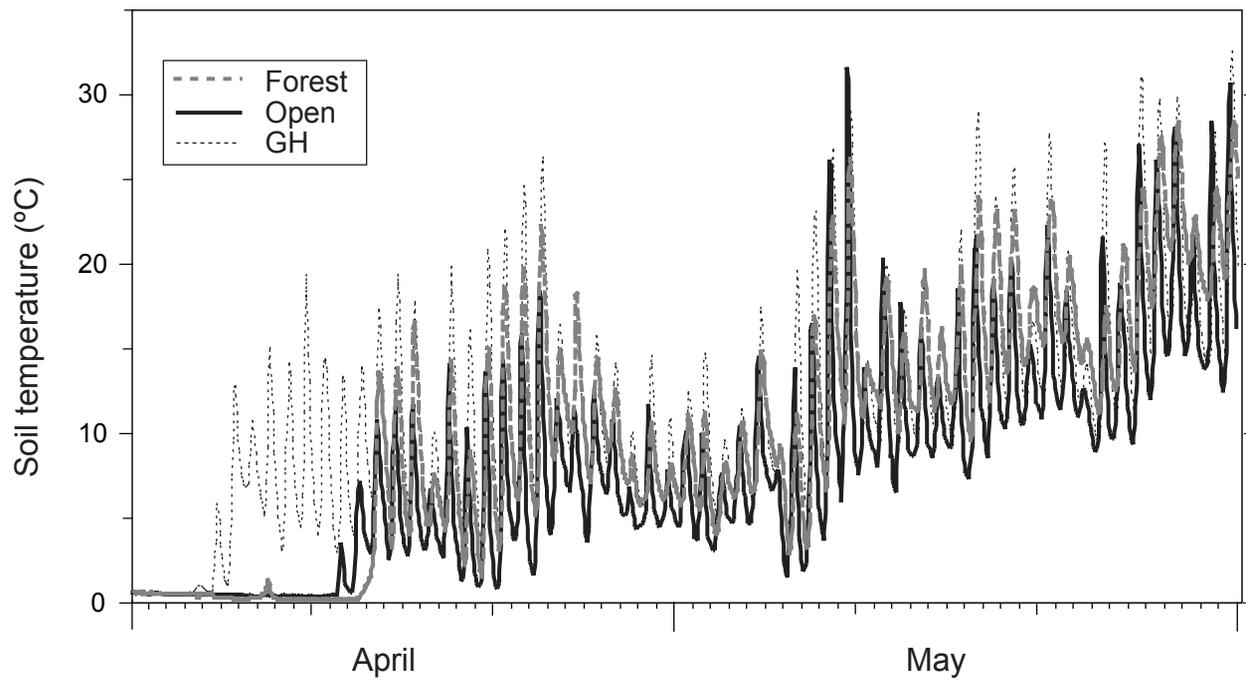


Fig. 5 (Sunmonu & Kudo)



Appendix Fig. 6 (Sunmonu & Kudo)