RESEARCH ARTICLE
Short Title: Kawai and Kudo—Life-history variation of alpine herbs

Variations in ramet performance and the dynamics of an alpine evergreen herb, *Gentiana nipponica*, in different snowmelt conditions

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PREMISE OF THE STUDY: Variation in demographic parameters reflects the life-history strategies of plants in response to specific environments. We aimed to investigate the intraspecific variation in life-history traits of a clonal alpine herb, *Gentiana nipponica*, in various snowmelt conditions.
METHODS: Individual ramets within genets accumulate leaves for 7–9 yr without shedding, and die after reproduction. We tested the physiological function of accumulated leaves for reproduction and monitored the ramet demography in early, intermediate, and late snowmelt populations over 3 yr. Then, we simulated ramet dynamics using the demographic parameters.
KEY RESULTS: Old leaves had a carbon storage function, and the initiation of reproduction depended on the amount of ramet leaves. Growth and reproductive performance were highest in the population with an intermediate snowmelt period. The early snowmelt population showed short persistence periods due to restricted growth and high mortality of the ramets. The late snowmelt populations showed slow growth, but high survival rate of the ramets, in which the ramet size at reproduction was smallest and fruit formation was often suppressed by the short growing period.
CONCLUSIONS: Limiting factors dictating the distribution of *G. nipponica* differed between the early and late snowmelt habitats. High mortality and restricted growth, because of the harsh environment, determine the distribution limit toward earlier snowmelt locations. By contrast, late snowmelt strongly limited fecundity because of the short period for fruit maturation. The difference in snowmelt time provides a clear gradient of selective forces that may promote local adaptation among neighboring populations.
KEY WORDS: demography; genet growth; Gentianaceae; growing season; leaf function; life-history variation; local adaptation; simulation; size dependency; snowmelt gradient.

Plant populations growing in different environmental conditions experience considerable variation in demographic processes, such as survival, growth, and fecundity (Hesse et al., 2008; Jacquemyn et al., 2010; Villellas and García, 2018). Such demographic variation might cause differences in population dynamics and reproductive outputs specific to local populations (e.g., Wesselingh et al., 1997; Franks et al., 2007; Williams et al., 2015). Therefore, comparison of the demographic variation of natural populations in different environmental situations represents an effective approach toward exploring the selective forces within individual habitats.

Alpine ecosystems in the mid-latitudes are often characterized by large variation in growing periods due to differences in the local timing of snowmelt (Kudo, 1991; Wipf and Rixen, 2010). Indeed, many studies have demonstrated that snowmelt pattern considerably influences plant growth (Wijk, 1986; Stinson, 2004; Wipf and Rixen, 2010; Mallik et al., 2011), leaf traits (Kudo et al., 1999; Welker et al., 2005), phenology (Galen and Stanton, 1995; Kudo, 2006, 2016; Wipf and Rixen, 2010), and reproductive success (Kudo, 2006, 2016; Cooper et al., 2011; Mallik et al., 2011). However, few studies have attempted to connect variation in these traits with the dynamics of local populations.

The selective forces affecting individual life-history traits might vary in response to local environmental heterogeneity in alpine ecosystems (Pluess and Stöcklin, 2005; Kawai and Kudo, 2011). In early snowmelt habitats, plants are exposed to longer durations of photosynthetic activity and growth; however, the risk of frost damage increases, if plants are exposed to cold climatic conditions early in the season after snowmelt (Inouye, 2008). In comparison, in late snowmelt habitats, the annual growth and reproductive activity of plants are often restricted because of the short growing periods and delayed phenological processes. However, lingering snow protects plants from spring frost and maintains moist soil conditions throughout the summer (Stinson, 2004; Wipf and Rixen, 2010; Cooper et al., 2011).

Among life-history traits, the timing of reproduction is a key strategy affecting lifetime fitness (Stearns, 1992). Plant size is often used as an estimate of resource availability at the time of reproduction (e.g., Reekie, 1997; Burd et al., 2006; Jacquemyn et al., 2010). Size at maturity is expected to be determined by a balance between the benefit of current reproduction and the associated costs for future survival and reproduction. Additionally, the size at maturity could be influenced by the resource availability of individual habitats (Jacquemyn et al., 2010; Brys et al., 2011; Villellas and García, 2018). Because the length of the growing season directly affects the annual carbon fixation and reproductive phenology of plants (Cooper et al., 2011; Sletvold and Ågren, 2015), growth, survival, fecundity, and the size dependency of reproduction may vary among populations with different growing periods (Haggerty and Galloway, 2011). For example, plants inhabiting late snowmelt locations may take longer to reach maturity than those inhabiting early snowmelt locations because of slower annual growth. However, plants may regulate the threshold size for reproduction in conditions with a restricted growing period (e.g., Sakai et al., 2003).

Evergreen habit and clonal growth are widely adopted properties for Arctic and alpine plant species (Callaghan and Collins, 1976). Although photosynthetic activity decreases with leaf age, the old leaves of evergreen plants often have a resource storage function for subsequent growth and reproduction (Jonasson, 1989, 1995; Karlsson, 1994). The amount of accumulated leaves, therefore, might constitute a resource pool for reproduction. In clonal plants, the multiplication of modular units (ramets) is important for maintaining populations, in addition to seed production by individual ramets within a genet (Hartnett and Bazzaz, 1983). Therefore, the foliage structure and dynamics of ramets are important demographic properties for understanding the response of clonal populations to environmental heterogeneity.

Gentiana nipponica Maxim. (Gentianaceae) is a perennial herb that forms small clonal patches and is widely distributed in the alpine snow meadows of northern Japan. Individual genets of this species are composed of multiple perennial ramets, in which evergreen leaves accumulate without shedding, and individual ramets die after a single reproductive event. This means that leaf number within a ramet represents plant size and resource availability if accumulated leaves act as a storage organ. As the life span and size of genets in each population are regulated by the balance between ramet survival, growth, and investment in reproduction, ramet dynamics across populations in various snowmelt

conditions could be monitored to detect how life-history traits of alpine plants respond to changes in the length of the growing season.

In the present study, we performed a combination of leaf-shading and partial-defoliation treatments to investigate the physiological function of accumulated leaves for reproduction. If old leaves act as a storage function alone, the shading of old leaves (leading to a reduction in exposure to photosynthetically active radiation) would not influence reproductive performance; however, the removal of old leaves would decrease fruit production because of resource limitation. Alternatively, if old leaves have both photosynthetic and storage functions, both defoliation and the shading treatment would reduce fruit production. In a field experiment, we tried to elucidate the factors that triggered the initiation of reproduction (i.e., threshold size for flowering at the ramet level and whether threshold size varied depending on habitat conditions). By monitoring ramet demography in multiple populations, we evaluated how plant growth strategies are affected by snowmelt conditions. Finally, we constructed genet growth models based on the field censuses of ramet dynamics in different snowmelt conditions. To understand the life-history strategies of clonal plants, the performance and dynamics of ramets must be assessed at the genet level, because the populations of clonal plants are maintained by both vegetative and sexual reproduction (e.g., Fischer and Kleunen, 2001). Therefore, our study aimed to answer the following questions: (1) Do old leaves accumulated within a ramet contribute as storage organs for reproduction? (2) Do the threshold size, growth rate, and survival rate of ramets differ among populations that experience different snowmelt conditions? (3) How does variation in ramet performance determine life-history properties at the genet level?

MATERIALS AND METHODS

Study site

This study was conducted in the central part of the Taisetsu Mountains, Hokkaido, northern Japan (43°33′N, 142°51–52′E) from 2005 to 2007. This mountainous area is characterized by warm, wet summers and cold, snowy winters. Mean air temperature in the summer season at 1700 m elevation is 3.0°C in May, 9.6°C in June, 11.6°C in July, 14.3°C in August, and 7.9°C in September (means for 2005–2007). Mean monthly precipitation is 120 mm in June, 124 mm in July, 300 mm in August, and 279 mm in September. The time of snow disappearance ranges from early June to mid-August. Snowfall begins in late September, and the ground is covered with snow by early October. In May 2005, four 20 × 20 m plots were established, one in an early snowmelt location (plot E), one in an intermediate snowmelt location (plot M), and two in late snowmelt locations (plots L-1 and L-2), which were located within 0.2–2.0 km of each other. The snowmelt date of individual plots during the observation period ranged from 27 May to 11 June in plot E, from 16 June to 25 June in plot M, from 15 July to 5 August in plot L-1, and from 28 July to 8 August in plot L-2 (Fig. 1; Appendix S1, see Supplemental Data with this article). The length of the growing period, calculated as the period during which the daily mean soil surface temperature exceeds 0°C, was 101–116 days in plot E, 87–96 days in plot M, 46–65 days in plot L-1, and 43–54 days in plot L-2 (Appendix S1).

Study plant

Gentiana nipponica commonly forms a small genet patch of several vertical stems (i.e., ramets). Ramets within a genet are loosely connected by thin stolons (Appendix S2). Individual ramets accumulate decussate evergreen leaves over several years without senescing. Plant height is <10 cm, and the diameter of genets ranges from 3 to 15 cm. Individual genet patches are spatially separated from each other and are easily distinguishable. Belowground parts (stolons and roots) are not well developed, which suggests they have very

little storage function. When the total leaf number reaches a critical level, flower primordia are formed in late summer of the year before flowering. Reproductive ramets typically have one to three (sometimes as many as five) campanulate protandrous purple flowers in a simple terminal or axillary cyme. This species is weakly self-compatible; seed set by artificial outcrossing was 76%, whereas seed set by artificial selfing was 19% (Y. Kawai and G. Kudo, unpublished data); however, pollinators are required to achieve fruit set, because of dichogamous flowering. Bumble bees (*Bombus* spp.) are the main pollinators, but dipteran and lepidopteran insects also visit this species (Mizunaga and Kudo, 2017). Ramets are monocarpic and die after reproduction, irrespective of fruiting success.

Leaf carbohydrates and nutrients

To examine the seasonal dynamics of leaf carbohydrates and nutrients within ramets, we measured leaf size, dry weight, and nitrogen content per leaf of reproductive ramets in plot M. This plot was located in the central part of the distribution range of *G. nipponica* along the snowmelt gradient, representing a typical growing habitat for this species. Sampling was conducted four times in 2005, during pre-flowering period soon after snowmelt on 25 June (except for leaf area), flowering period on 10 August, fruiting period on 8 September, and the late-growing period on 26 September, at which point floral buds had started to develop (previous year of flowering). We sampled 15–20 ramets at each time point by collecting a single ramet from individual genets to produce independent sample points. The opposite and decussate leaves on each ramet were divided into clusters of four leaves from top to bottom, and the five top clusters (20 leaves) were used for the analysis. After scanning all leaves with an image scanner connected to a personal computer, the individual leaf area, except for the leaves soon after snowmelt, was measured using the public domain NIH Image software (U.S. National Institutes of Health; http://rsb.info.nih.gov/nih-image/). After 48 h of drying the leaves at 70°C, the dry weight of each cluster of four leaves was measured. Subsequently, the total nitrogen content per leaf (hereafter Leaf N) was measured for each cluster using a Vario EL C-H-N analyzer (Elementar, Langenselbold, Germany). Five clusters were grouped as upper leaves (first to third top clusters) and lower leaves (fourth and fifth clusters) for analysis.

To examine the potential role of old leaves in resource storage for reproduction, 45 genets of *G. nipponica* were selected in plot M during late June 2006 and were randomly assigned to three groups (15 genets per group): control, shading treatment, and defoliation treatment. The treatments were conducted on a single reproductive ramet per genet. To prevent potential resource transport through physiological connection between ramets, we severed all stolon connections from a focal reproductive ramet within a genet. In the shading treatment, the lower half of all leaves were wrapped in aluminum foil to prevent photosynthesis until fruit maturation in mid-September. In the defoliation treatment, the lower half of all leaves within ramets were artificially removed using tweezers. All flowers were cross-pollinated by hand to prevent pollen limitation for fruit production. During the fruiting period, all fruits were harvested and their seeds were counted.

Monitoring of ramet dynamics

We established a 5 × 5 m quadrat in each plot (E, M, L-1, L-2) soon after snowmelt in 2005. In each quadrat, 50 genets of *G. nipponica* were randomly chosen, and all ramets within the genets were labeled with small numbered tags (Appendix S2). Then, we recorded the growth status (vegetative, reproductive, dead) and the number of leaves on all ramets. Measurements were conducted three times during the growing periods in 2005 and 2006 (during the pre-flowering period soon after snowmelt, flowering period, and fruiting period), and two times in 2007 (during the pre-flowering and flowering periods). Annual leaf formation was defined

as the increase in the number of leaves from the previous to the current pre-flowering period (2005–2006 and 2006–2007). Newly emerged ramets were also marked. For reproductive ramets, the number of flowers was recorded. Because ramets retained almost all leaves produced without shedding, we were able to use leaf number as a representative of ramet size.

Data analysis

All statistical analyses were performed using an open source system, R version 3.5.0 (R Core Team, 2018). We implemented generalized linear mixed-effect models (GLMMs) using the R function "glmer" or "lmer" in the library "lme4," and generalized linear models (GLM) using the R function "glm" for the analyses. To find the best-fitting models, all full models were compared with all possible model subsets based on Akaike's information criterion (AIC; Burnham and Anderson, 2003).

In the experiment on the physiological function of accumulated leaves, seasonal changes in leaf dry weight, nitrogen content, and leaf area were analyzed in relation to leaf position by a GLMM postulating a Gaussian error distribution. In the model, sampling time (previous year of flowering, pre-flowering, flowering, fruiting stage), leaf position within a ramet (upper, lower), and their interaction were incorporated as explanatory variables, and ramet ID was incorporated as random intercepts. The total number of seeds within a ramet was compared among leaf treatments by a GLM postulating a Poisson error distribution, in which treatment (control, shading, defoliation) and number of flowers per ramet were incorporated as explanatory variables.

Size-dependent flowering probability and flower number per ramet were analyzed by a GLMM postulating a binomial error distribution with a logit-link function and a Poisson error distribution, respectively. Full models included plot (E, M, L-1, and L-2), ramet size (leaf number), and their interaction as explanatory variables, and genet ID repeatedly measured over years was incorporated as a nested random effect by year.

We compared annual leaf formation (as an index of ramet growth) among plots over 2 yr (2005 and 2006) by a GLMM postulating a Poisson error distribution. Plot, ramet size (number of leaves in the previous year), and their interaction were incorporated as explanatory variables, and genet ID repeatedly measured over years was incorporated as a nested random effect by year in the full model. Reproductive ramets were excluded from the analysis because they did not produce additional leaves.

We applied Cox proportional hazards regression to compare the survival rate of ramets among plots (Cox, 1972). We recorded the fate of 1555 vegetative ramets over 3 yr from snowmelt in 2005 to the flowering period of 2007. The instantaneous probability of mortality for individual ramets at time t was computed based on the combination of a nonparametric baseline (h_0) function and an exponential function of k:

$$h_i(t) = h_0(t)\exp(\beta_1\chi_{i1} + \cdots + \beta_k\chi_{ik})$$

where i is ramet identity, χ_{ik} is measurement trait k of ramet i, and β_k is a coefficient of χ_{ik}. Plot, ramet size, and their interaction were included as covariate variables, and genet ID was incorporated as random intercepts. We used R function "coxph" in the library "survival" to estimate the β coefficient for each model covariate and to test the null hypothesis of $\beta = 0$ with a χ^2 statistic from the approximation in the maximum partial likelihood method. Negative coefficient values were associated with decreasing mortality, and positive values were associated with increasing mortality.

Genet properties were analyzed by a GLMM postulating a Poisson error distribution for the total number of ramets (genet size) and the number of newly produced ramets per genet (genet growth), and by a GLMM postulating a binomial error distribution with a logit-

link function for the proportion of reproductive ramets per genet (reproductive activity). In these models, plot (E, M, L-1, L-2) was incorporated as an explanatory variable, and genet ID repeatedly measured over years was incorporated as a nested random effect by year.

Simulation of ramet and genet growth

Because individual ramets exist for several years, it was difficult to measure ramet age directly from observations over a few years in the field. Thus, we constructed a model to estimate the ramet age at reproduction (reproductive age model) using three parameters: (1) survival rate, (2) flowering probability, and (3) growth rate (i.e., annual leaf formation). The effect of yearly variation was included in the simulation using parameters obtained using the GLMMs for 2005 and 2006 separately. Full models contained plot, ramet size (leaf number), and their interaction as explanatory variables, and genet ID as random intercepts. In order to simulate yearly variation in snowmelt time, the parameters of 2005 and 2006 were applied randomly. We assumed that each ramet started from two leaves at age 1 yr, as observed in the field. The simulation process was as follows:

- Step 1.1: Survival (go to Step 1.2) or death (endpoint) at a certain age.
- Step 1.2: Flowering (endpoint, record the age and size) or not flowering (go to Step 1.3).
- Step 1.3: Addition of leaves within a ramet based on the growth parameter, and return to Step 1.1 with one age addition.

Individual ramets continue to grow (i.e., leaf accumulation) from year to year in accordance with size-specific parameters until the endpoint, which was either death before flowering or senescence after reproduction. We obtained the survival rate of ramets until reaching the reproductive stage, and mean ramet size (leaf number) and age at reproduction from simulations with 10,000 runs (i.e., the fates of 10,000 ramets). Mean ramet size and age at reproduction of successfully surviving ramets until flowering in the simulation were compared among plots using a GLM postulating a Gaussian error distribution.

Furthermore, we constructed a genet growth model to estimate the ramet dynamics within genets based on the observed ramet performance in each population. Genet growth means the cumulative number of ramets produced in each genet from year to year. In addition to the three parameters used in the reproductive age model (i.e., survival rate, flowering probability, and growth rate), we used the parameter of ramet recruitment (i.e., estimated number of newly produced ramets within a genet) using a GLM postulating a Poisson error distribution, in which plot, genet size, and their interaction were included as explanatory variables. GLM was conducted separately for each year (2005 and 2006). These four parameters of 2005 and 2006 (see Appendix S3) were randomly applied for the simulation of genet growth every year. In the simulation, we assumed that individual genets start from a single ramet with two leaves, and that newly recruited ramets start to grow from two leaves. The simulation process was as follows:

- Step 2.1: Recruit of new ramets within a genet.
- Step 2.2: Survival, flowering, and leaf production of ramets within a genet, based on the reproductive age model (Steps 1.1–1.3).
- Step 2.3: Return to Step 2.1 or death of all ramets (endpoint).

The number of ramets of individual genets fluctuates from year to year, reflecting the frequencies of recruitment and disappearance of existing ramets until the death of all ramets. Simulations were iterated with 100 runs and were repeated 100 yr. Because none of the

genets in E survived after 100 yr of simulations (see below), survival rate, cumulative numbers of total and reproductive ramets, and the proportion of ramets reaching the reproductive stage were compared at both the 20th and the 100th year of simulations.

RESULTS

Leaf carbohydrates and nutrients

The weight of the upper leaves (first to third positions) on reproductive ramets increased by approximately 20–30% in the early season after snowmelt and decreased rapidly toward midsummer when flowering occurred (Fig. 2A; for statistical results, see Appendix S4). The weight of the lower leaves (fourth and fifth positions) continuously decreased as the season progressed, indicating resource translocation from the lower to upper leaves. Leaf N was significantly lower in the lower leaves than the upper leaves. There was little seasonal fluctuation in leaf N of the lower leaves, whereas that of the upper leaves increased highly on 25 June and then decreased (Fig. 2B; for statistical results, see Appendix S4). The upper leaves increased in size significantly during the flowering and fruiting stages, whereas the seasonal trend of the lower leaves was unclear (Fig. 2C; for statistical results, see Appendix S4).

Seed production was affected by the leaf treatment. There were no differences in the number of seeds per ramet between the control (23.5 ± 21.6; mean ± SD) and shading (20.5 ± 18.9, $z = -1.63$, $P = 0.10$) treatments. However, the removal of old leaves significantly reduced number of seeds (10.0 ± 15.3, $z = -7.95$, $P < 0.001$). The effect of flower number was excluded from the explanatory variables in the best-fitting model.

Ramet properties

Flowering occurred from late July to mid-August in plots E and M, whereas the major flowering period of the late snowmelt populations (plots L-1 and L-2) varied from late August to mid-September (Fig. 1). Because the snowmelt time in plots L-1 and L-2 was very late (early August) in 2005 and 2006, flowering occurred in September in these populations, and plants could not set any fruits because snow covered the ground in late September before fruiting in these years.

The flowering probability of individual ramets was strongly size dependent for all plots (Fig. 3). This trend was more apparent in plots L-1 and L-2 (significant interactions between plot and leaf number; Table 1), indicating smaller ramet size at flowering in the late snowmelt populations. The number of flowers per ramet was greatest in plot M, but there were no significant differences among the other plots (Table 1). The mean number of flowers per ramet ranged from 2.2 to 2.6 in plot M, and from 1.3 to 2.2 in other plots during the observation years (Appendix S5). Size dependency and interactions between plot and leaf number were excluded in the best-fitting model.

Annual leaf formation was greatest in plot M ($z = 7.54$, $P < 0.001$) and lowest in plot L-1 ($z = -1.88$, $P = 0.060$; see Appendix S5 and S6). Initial ramet size was negatively correlated with annual leaf production ($z = -2.53$, $P = 0.012$), indicating that larger ramets tended to produce fewer leaves than smaller ramets (Appendix S6).

The survival rate of ramets clearly depended on snowmelt time, which was lowest in plot E, followed by plots M, L-1, and L-2 (0.46, 0.54, 0.66, and 0.75, respectively; Fig. 4). There was a positive correlation between the leaf number and ramet mortality ($z = 8.50$, $P = 0.003$; see Appendix S7), indicating higher mortality in larger ramets for all plots.

Genet properties

Individual genets were composed of 6.5–15.2 ramets, on average, across plots and observation years (Appendix S5). The number of ramets per genet was smallest in plot E,

intermediate in plot L-1 ($z = 3.65$, $P < 0.001$) and plot L-2 ($z = 3.42$, $P < 0.001$), and largest in plot M ($z = 6.80$, $P < 0.001$). The number of newly produced ramets was larger in plot M ($z = 3.51$, $P < 0.001$) than in the other plots. The proportion of reproductive ramets per genet was higher in plot L-1 ($z = 3.14$, $P = 0.002$) and plot L-2 ($z = 2.18$, $P = 0.029$) than in plots E and M (for details of the data, see Appendix S5). These results indicated that ramet production and the reproductive activity of genets were constrained in the earliest snowmelt population (plot E), whereas higher reproductive activity was retained in the late snowmelt populations (plots L-1 and L-2).

Simulation of ramet and genet dynamics

Simulation results of the age–size relationship of reproductive ramets are shown in Figure 5, in which we randomly selected 100 ramets from 10,000 repetitions and only flowering ramets were plotted for visual clarity (parameters used for the simulation are shown in Appendix S3). Only 17% of ramets reached the reproductive stage in plot E, whereas 37%, 44%, and 64% of ramets reached the reproductive stage in plots M, L-1, and L-2, respectively. The reproductive ramet size in plot M (30.8 ± 7.2 leaves; $t = 4.51$, $P < 0.001$) was larger than that of plot E (28.4 ± 7.2; intercept), whereas the reproductive ramets of plot L-1 (23.5 ± 5.0) and plot L-2 (25.7 ± 4.9) were smaller than those of plot E ($t = -9.33$ and $t = -5.32$, $P < 0.001$, respectively). By contrast, the estimated ramet age at flowering was youngest in plot M (7.0 ± 1.8 yr old; $t = -10.88$, $P < 0.001$). Ramets in plot E required the longest time to reach flowering (9.1 ± 2.6 yr old; intercept), followed by plot L-1 (8.4 ± 2.3 yr old; $t = -4.08$, $P < 0.001$) and plot L-2 (8.3 ± 2.0 yr old; $t = -4.39$, $P < 0.001$).

The simulation results of genet growth during 100 yr are shown in Figure 6, in which only <50 reproductive genets of 100 repetitions are shown for visual clarity (parameters are shown in Appendix S3). The survival of genets varied highly among the plots: genets in plot E had the shortest persistence period, and most genets disappeared by 50 yr in the simulation. In plot L-2, many genets existed by 40 yr, but only <10% of genets survived >100 yr. By contrast, genets with more intermediate snowmelt times had longer survival, in which 23% of genets in plot M and 35% of genets in plot L-1 survived in simulations for 100 yr. When genet dynamics were compared for a shorter time scale (i.e., 20 yr from establishment; Table 2), the survival rates of genets ranged from 22% (plot E) to 79% (plot L-1), the cumulative production of ramets per genet ranged from 3.8 (plot E) to 13.5 (plot M), and the cumulative production of reproductive ramets ranged from 0.5 (plot E) to 3.8 (plot M). The proportion of ramets reaching the reproductive stage during 100 yr increased in the later snowmelt plots: 25% in plot E, 40% in plot M, 48% in plot L-1, and 67% in plot L-2.

DISCUSSION

Alpine environments are composed of diverse microhabitats that are largely related to local snowmelt conditions (Wipf and Rixen, 2010), and the heterogeneity of ecological situations may promote the differentiation of life-history traits among neighboring populations. Our results demonstrate that the growth and demographic properties of *G. nipponica* varied significantly among local populations in different snowmelt conditions. Older leaves seemed to store carbohydrate, and the initiation of reproduction depended on the amount of leaves accumulated within a ramet. Growth and reproductive performance were highest in the population in intermediate snowmelt conditions. The early snowmelt population showed small genet size and shorter persistence period because of restricted growth and high mortality of the ramets. The late snowmelt populations showed slow growth but high survival of ramets, and ramet size at reproduction was smallest and fruit set was limited by the short growing period. Therefore, the life-history traits of this species depended strongly on the snowmelt time experienced by individual populations.

The resource storage function of evergreen leaves has been reported in several tundra plants (Chapin, 1980; Jonasson, 1989). Because the assimilative function of old leaves was negligible in our experiment, carbohydrates stored in the old leaves likely supported the development of young leaves in the reproductive year, as reported in other evergreen species (Jonasson, 1989), whereas young leaves appeared to act as the assimilative organs for flower and fruit production. The increments in mass and area of young leaves contributed toward enhancing photosynthetic products during the pre-flowering period (Karlsson, 1994). In contrast to the carbon supply from old leaves, the increase in nitrogen content of young leaves during the pre-flowering period might result from nitrogen uptake by the roots. Alpine plants often increase nitrogen early in the growing season, because the release of soluble nutrients by microbial activity is highest soon after snowmelt (Legay et al., 2013). Because leaf nitrogen content is often related to photosynthetic ability (Evans, 1989), high leaf nitrogen content can facilitate the high photosynthetic activity of young leaves during the pre-flowering period. Rapid decreases in leaf weight and nitrogen content in young leaves during the flowering period indicated a large demand for carbohydrate and nitrogen for reproduction (Jonasson, 1989; Karlsson, 1994; Bausenwein et al., 2001).

As expected, the annual growth of ramets and new ramet production were restricted when the growing period was limited. However, the growth and recruitment of ramets were also restricted in the early snowmelt population. Earlier snowmelt might expose plants to higher risk of frost damage (Inouye, 2008). From late May to early June, the minimum air temperature sometimes decreased below zero, and solar radiation was strong on clear days. This situation enhances the risk of photoinhibition in alpine plants (Germino and Smith, 2000). Moreover, the extended growth, because of early snowmelt, might lead to drought stress for alpine plants later in the season (Berdanier and Klein, 2011). Indeed, the soil moisture in the early snowmelt habitat decreased more intensively in the middle of summer than that of the late snowmelt habitat in our study area (Y. Kawai and G. Kudo, unpublished data). Therefore, physiological damage by frost and photoinhibition early in the season and/or drought stress late in the season might overwhelm the positive effect of an extended growing period in the early snowmelt population, resulting in low growth rates and high mortality.

In alpine environments, late flowering often causes seed maturation to fail, because of the short growing period (Kudo, 1991; Molau, 1993; Cooper et al., 2011). This phenomenon is especially common in snowbed plant species inhabiting particularly late snowmelt locations (Kudo, 1991). Actually, the developing seeds of *G. nipponica* in the late snowmelt populations failed to mature on three occasions during five observation years (Kawai and Kudo, 2011). In our study area, snowfall usually begins in late September, when plants are frequently exposed to freezing temperature, and the ground is covered with snow by early October. Therefore, seed production in the late snowmelt populations is successful only during occasional long summers with early snowmelt. Because of restricted seed production, the relative importance of vegetative growth and ramet production might be higher in short-growing-period conditions (Bienau et al., 2016). Despite slow ramet growth, the high survival rate of ramets and the long persistence of genets contribute to the maintenance of populations in the late snowmelt habitat.

Although the shift to the reproductive stage depended on the number of leaves accumulated within a ramet, the threshold size for reproduction varied among populations. Flowering at a smaller size in the late snowmelt populations might enhance the probability of reaching the reproductive stage, despite the low growth rate. The life-history theory predicts that optimal size and age at maturity are determined by a trade-off between current reproductive output and future cost of survival and reproduction (Stearns, 1992). Sakai et al. (2003) investigated the variation in life-history traits of a sub-alpine conifer, *Abies mariesii*, located at different altitudes and reported that trees advanced reproductive schedules toward

smaller size and younger age at higher sites, where harsher environments bring about lower growth rate and higher mortality. Although the mortality of ramets was relatively low in the late snowmelt populations in our study, smaller threshold size for reproduction may increase the production of reproductive ramets within a genet even in conditions producing a slow growth rate. Because seed maturation in the late snowmelt populations was possible only in years with a longer snow-free period, continuous production of reproductive ramets may enhance the chance to catch a good year.

The smaller size of reproductive ramets in the late snowmelt populations may be attained with fewer flowers produced per ramet. Because the flowering of *G. nipponica* progresses sequentially within an inflorescence, an increase in flower number results in an extended flowering period for the ramet, which is likely to increase the proportion of fruits that fail to mature in short-growing-period conditions. Therefore, the production of more flowers may not substantially increase the fitness in the late snowmelt populations, at least in typical years.

The simulation of genet growth indicated large variations in demographic properties among the populations (Fig. 6). The size of reproductive ramets was maximal in the intermediate snowmelt population (plot M), where rapid ramet growth and recruitment offset low ramet survival. This place can be regarded as the optimal habitat for *G. nipponica* among the plots. In the simulation of the early snowmelt population (plot E), almost all ramets died before flowering because of low growth, survival, and recruitment rates of ramets. Thus, this habitat appeared to represent an earliest margin of the niche space in relationship to the snowmelt environment. By contrast, the simulation results for the late snowmelt populations (plots L-1 and L-2) suggested that individual ramets have a high probability of reaching the reproductive stage and the long persistence of genets owing to their high survival rate and small reproductive size. However, the failure of seed production, because of the shorter growing period, is likely to constitute a latest margin of the snowmelt niche space for this species.

In conclusion, the regulation of leaf accumulation within a ramet is a fundamental mechanism that regulates the reproductive performance and longevity of ramets, leading to various population dynamics in different snowmelt conditions for *G. nipponica*. Intraspecific variation in leaf life span has been reported for many alpine plant species along the snowmelt gradient (Kudo et al., 1999, 2001). However, the relationships between leaf life span and demographic properties have rarely been studied at the intraspecific level. The present study demonstrated the linkage between leaf life span and life-history strategy of an alpine plant inhabiting a range of snowmelt conditions. Several studies have demonstrated that natural populations harbor substantial genetic variation in life-history traits, such as the threshold size for reproduction (Wesselingh et al., 1997) and flowering time (Franks et al., 2007; Haggerty and Galloway, 2011). Although we cannot address the contributions of genetic variation and environment to the observed variations in life-history traits in this study, differences in the flowering period restricted pollen exchange among populations inhabiting different snowmelt conditions (phenological isolation of gene flow), and this might maintain the local genetic variation in life-history traits (Kudo, 2006). Indeed, significant genetic differentiation was detected among *G. nipponica* populations along the snowmelt gradient in the same area (Hirao and Kudo, 2004). Furthermore, our previous transplantation experiment revealed the possibility of genetic variation in flowering phenology between the early and late snowmelt populations in this species (Kawai and Kudo, 2011). These results strongly suggested the existence of different selective forces along the snowmelt gradient in alpine environments (i.e., early-season frost damage and mid-season drought stress) in the early snowmelt habitat vs. limitation of growing period in the late snowmelt habitat. These heterogeneous selective forces may promote local adaptation of the life-history traits among

neighboring populations of alpine plants. At the same time, rapid modification of snowmelt conditions by global warming should disturb the inherent ecological situations of local habitats (e.g., Winkler et al., 2018). The predicting plant responses to these climatic changes, such as earlier snowmelt or later onset of winter, may be different between early and late snowmelt populations. Whether local populations can adapt to the changing conditions is a crucial issue for future study.

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DATA ACCESSIBILITY
The data set used by this article is available in the Dryad Digital Repository: doi:10.5061/dryad.tg20p4k.

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FIGURE 1. Reproductive phenology of *Gentiana nipponica* in each plot, 2005–2007. Dots, closed columns, and open columns indicate snowmelt time, flowering period, and fruiting period, respectively. Crosses indicate no flowering or fruiting within a plot.

FIGURE 2. Seasonal changes in (**A**) dry weight per leaf, (**B**) nitrogen content per leaf, and (**C**) individual leaf area of upper and lower leaves within reproductive ramets. Sampling was conducted four times: previous year of flowering, pre-flowering period in current year (except for leaf area), flowering period, and fruiting period. Circles and squares indicate upper leaves and lower leaves, respectively, with SE values. *** $P < 0.001$, ** $P < 0.01$ by GLMM. See Appendix S3 for statistical results.

FIGURE 3. Frequency of the number of leaves per ramet in each plot in 2005. White and gray bars indicate vegetative and reproductive ramets, respectively.

FIGURE 4. Survival of ramets from the snowmelt in 2005 to the flowering season in 2007 for each plot with a Cox proportional hazards regression model (M = soon after snowmelt. F = flowering period, S = fruiting period). ns $P > 0.05$, * $P < 0.05$, *** $P < 0.001$. For statistical results, see Appendix S6.

FIGURE 5. Predicted ramet size (leaf number) and age at flowering of *Gentiana nipponica* in each plot. For visual clarity, the fates of 100 ramets were randomly selected from 10,000 repetitions of simulation, and only the results for flowering ramets are plotted. Arrows indicate mean values.

FIGURE 6. Simulation results of genet growth of *Gentiana nipponica* in each plot. Cumulative numbers of reproductive ramets produced by individual genets during 100 yr are indicated. Only <50 genets of 100 repetitions are plotted. Individual genets started from one ramet with two leaves. The disappearance of lines indicates the death of genets.

TABLE 1. Comparisons of size-dependent flowering probability and the number of flowers per ramet among plots by GLMM. Plot (E, M, L-1, L-2) and leaf number per ramet are included as explanatory variables. NA = variable excluded in the best-fitting model. Significant *P* values are in bold.

	Flowering probability				Flower number			
Variable	Estimate	SE	*z*	*P*	Estimate	SE	*z*	*P*
Intercept (E)	−7.05	0.57	−12.39	**<0.001**	0.52	0.11	4.85	**<0.001**
Plot M	0.30	0.62	0.48	0.63	0.34	0.11	3.19	**0.001**
Plot L-1	−1.37	0.72	−1.91	**0.06**	0.10	0.11	0.91	0.36
Plot L-2	−1.12	0.72	−1.56	0.12	0.10	0.11	0.87	0.38
Leaf number	0.19	0.02	9.57	**<0.001**	NA			
M × Leaf no.	0.005	0.02	0.20	0.84	NA			
L-1 × Leaf no.	0.13	0.03	4.29	**<0.001**	NA			
L-2 × Leaf no.	0.10	0.03	3.39	**<0.001**	NA			

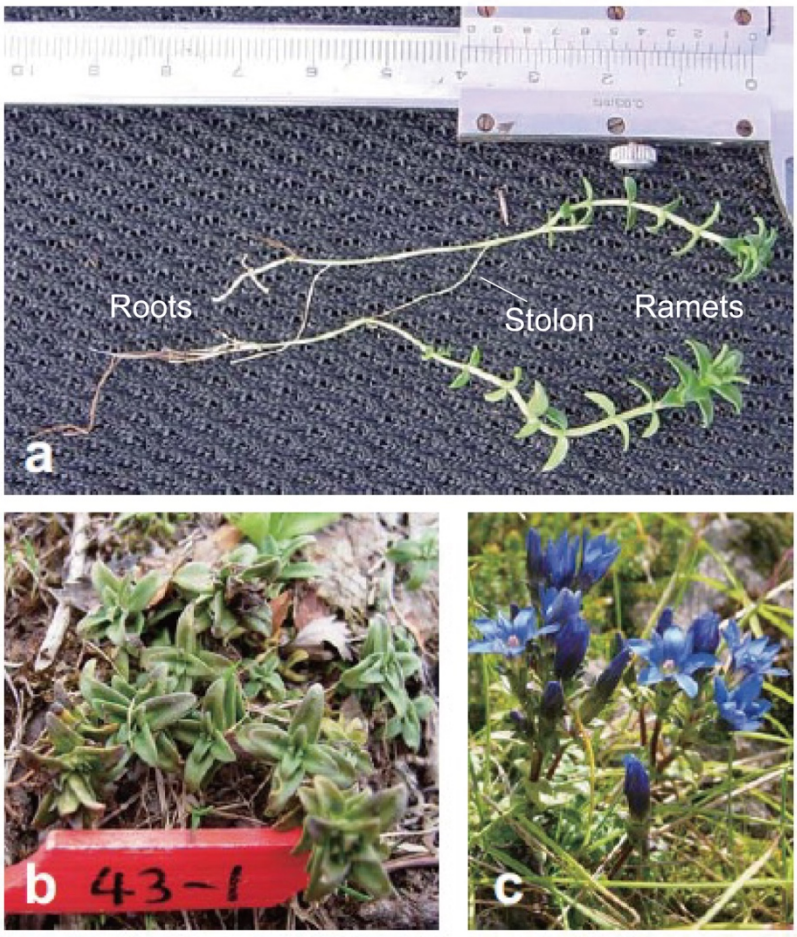
TABLE 2. Simulation results of genet growth over 20 yr in each plot ($N = 100$ genets). The number of surviving genets and the cumulative numbers of total and reproductive ramets per genet (mean ± SD) are indicated.

Plot	Surviving genets (*n*)	Total ramets (*n*)	Reproductive ramets (*n*)
E	22	3.8 ± 4.3	0.5 ± 0.8
M	75	13.5 ± 7.0	3.8 ± 2.5
L-1	79	10.7 ± 4.8	3.2 ± 2.1
L-2	56	5.8 ± 4.3	2.7 ± 2.0

Appendix S1. Plot characteristics of *Gentiana nipponica* populations in the Taisetsu Mountains, Hokkaido, northern Japan. Snowmelt time means the first date when daily mean soil-surface temperature increased above 0°C, and growing season length means the period (day) during which daily mean soil-surface temperature retained above 0°C. Soil surface temperature was measured with automatic loggers in each plot.

Plot	Elevation		Snowmelt time			Growing season length (day)		
		Year	2005	2006	2007	2005	2006	2007
E	1890 m		9 June	27 May	11 June	103	116	101
M	1840 m		25 June	16 June	25 June	87	96	87
L-1	1790 m		5 August	17 July	3 August	46	65	48
L-2	1800 m		8 August	28 July	3 August	43	54	48

Appendix S2. Pictures of *Gentiana nipponica*. **a**: ramet structure, **b**: genet composed of several ramets, **c**: flowering ramets



Appendix S3. GLMM results for survival, flowering probability, and growth rate for the estimations of dynamics and reproductive age of ramets in each plot. Plot (E, M, L-1, L-2), leaf number per ramet (ramet size), and their interactions are incorporated as explanatory variables, and genet ID as a random factor. In addition, GLM result of ramet recruitment was shown to estimate dynamics of genet growth in each plot. Plot, previous ramet number, and their interactions are incorporated as explanatory variables.

Property	Year	Variable	Coefficient	SE	*z* value	*P* value
Survival rate	2005	Intercept (Plot E)	1.041		3.88	<0.001
		Plot M	1.098		3.67	<0.001
		Plot L-1	1.515		4.61	<0.001
		Plot L-2	2.231		5.97	<0.001
		Leaf no.	0.033		2.49	0.01
	2006	Intercept (Plot E)	1.480		4.39	<0.001
		Plot M	-0.215		-0.56	0.57
		Plot L-1	0.291		0.73	0.46
		Plot L-2	0.763		1.88	0.06
		Leaf no.	0.011		0.90	0.37
Flowering probability	2005	Intercept (Plot E)	-6.736	0.84	-8.02	<0.001
		Plot M	0.550	0.99	0.56	0.58
		Plot L-1	-0.591	1.14	-0.52	0.60
		Plot L-2	-1.655	1.20	-1.38	0.17
		Leaf no.	0.184	0.03	5.83	<0.001
		M ×Leaf no.	-0.010	0.04	-0.25	0.81
		L-1×Leaf no.	0.086	0.05	1.80	0.07
		L-2×Leaf no.	0.155	0.05	3.04	0.002
	2006	Intercept (Plot E)	-7.861	1.01	-7.76	<0.001
		Plot M	-0.422	1.21	-0.35	0.73
		Plot L-1	-2.467	1.40	-1.76	0.08
		Plot L-2	-2.401	1.54	-1.56	0.12
		Leaf no.	0.248	0.04	6.44	<0.001
		M ×Leaf no.	0.010	0.05	0.20	0.84
		L-1×Leaf no.	0.180	0.06	3.02	0.003
		L-2×Leaf no.	0.123	0.06	1.93	0.05
Annual leaf formation	2005	Intercept (Plot E)	1.171	0.10	11.75	<0.001
		Plot M	0.300	0.12	2.49	0.01
		Plot L-1	-0.409	0.15	-2.74	0.006
		Plot L-2	0.119	0.13	0.89	0.37
		Leaf no.	-0.009	<0.01	-1.5	0.13
		M × Leaf no.	0.009	<0.01	1.17	0.24
		L-1×Leaf no.	0.004	0.01	0.37	0.71
		L-2×Leaf no.	-0.017	<0.01	-1.86	0.06

	2006	Intercept (Plot E)	1.236	0.12	11.93	<0.001
		Plot M	0.411	0.14	3.28	0.001
		Plot L-1	0.238	0.16	1.68	0.09
		Plot L-2	-0.111	0.16	-0.78	0.43
		Leaf no.	-0.004	<0.01	-0.68	0.50
		M ×Leaf no.	0.002	<0.01	0.26	0.79
		L-1×Leaf no.	-0.010	<0.01	-1.08	0.28
		L-2×Leaf no.	0.018	<0.01	1.97	0.05
Ramet recruitment	2005	Intercept (Plot E)	-1.427		-4.94	<0.001
		Plot M	1.041		3.10	0.002
		Plot L-1	0		0	1
		Plot L-2	-0.090		-0.21	0.84
	2005	Intercept (Plot E)	-0.624		-1.40	0.16
		Plot M	0.314		0.63	0.53
		Plot L-1	0.813		1.43	0.15
		Plot L-2	-0.122		-0.22	0.83
		Previous ramet no.	0.052		0.86	0.38
		M ×Ramet no.	0.005		0.08	0.94
		L-1×Ramet no.	-0.122		-1.71	0.09
		L-2×Ramet no.	0.022		0.34	0.74

Appendix S4. GLMM results of seasonal changes in dry weight per leaf (mg), nitrogen content per leaf (μg), and leaf area (cm^2). Leaves were sampled four times (previous year of flowering, pre-flowering, flowering and fruiting), and classified as upper (top to 12 leaves) and lower (top 13 to 20 leaves) positions within ramets.

Property	Variable	Coefficient	SE	*t* value	*P* value
Leaf weight	Intercept*	1.22	0.08	15.07	**<0.001**
	Pre-flowering	0.31	0.10	2.97	**0.004**
	Flowering	-0.06	0.11	-0.52	0.61
	Fruiting	-0.10	0.11	-0.90	0.37
	Lower leaves	-0.05	0.07	-0.65	0.52
	Pre-flowering ×Lower leaves	-0.43	0.10	-4.19	**<0.001**
	Flowering ×Lower leaves	-0.06	0.10	-0.53	0.60
	Fruiting ×Lower leaves	-0.12	0.11	-1.11	0.27
Leaf N	Intercept*	23.44	1.58	14.81	**<0.001**
	Pre-flowering	8.07	2.05	3.94	**<0.001**
	Flowering	-1.57	2.30	-0.68	0.50
	Fruiting	-3.03	2.44	-1.24	0.21
	Lower leaves	-6.44	1.54	-4.17	**<0.001**
	Pre-flowering ×Lower leaves	-7.37	2.03	-3.64	**<0.001**
	Flowering ×Lower leaves	-0.16	2.22	-0.07	0.94
	Fruiting ×Lower leaves	2.14	2.46	0.87	0.39
Leaf area	Intercept*	0.18	0.01	16.90	**<0.001**
	Flowering	0.03	0.02	2.13	**0.037**
	Fruiting	0.04	0.02	2.26	**0.027**
	Lower leaves	-0.004	0.01	-0.49	0.63
	Flowering ×Lower leaves	-0.03	0.01	-2.22	**0.032**
	Fruiting ×Lower leaves	-0.05	0.01	-3.73	**<0.001**

* Intercept includes “Previous year of flowering” and “Upper leaves”

Appendix S5. Properties of ramets and genets of *Gentiana nipponica* in each plot during the observation periods. Mean ± SD.

Year	Plot	Vegetative ramet		Reproductive ramet			Genet			
		Annual leaf production	*N*	Leaf No.	Flower No.	*N*	Ramet No.	Reproductive ramet (%)	Annual ramet production	*N*
2005	E	2.3 ± 2.5	257	27.1 ± 6.9	1.8 ± 0.7	26	8.1 ± 3.5	7.8 ± 11.2	0.54 ± 0.9	50
	M	3.8 ± 2.9	491	23.6 ± 6.0	2.6 ± 1.0	39	14.0 ± 8.4	9.2 ± 17.1	1.08 ± 1.5	50
	L-1	1.6 ± 1.8	365	22.4 ± 3.0	2.1 ± 1.0	51	10.2 ± 5.9	12.0 ± 10.5	0.48 ± 1.0	50
	L-2	2.3 ± 2.2	382	23.2 ± 3.8	2.2 ± 0.9	67	10.1 ± 4.6	16.2 ± 10.5	0.46 ± 0.9	50
2006	E	3.4 ± 3.1	215	27.2 ± 5.4	1.7 ± 0.9	36	6.7 ± 2.9	11.3 ± 12.6	1.44 ± 1.9	49
	M	4.3 ± 3.4	352	25.4 ± 5.6	2.3 ± 1.3	57	13.7 ± 9.1	7.4 ± 10.3	3.36 ± 3.6	50
	L-1	3.7 ± 2.8	291	23.5 ± 4.0	1.8 ± 1.1	70	10.4 ± 6.0	13.6 ± 12.1	1.58 ± 1.9	50
	L-2	3.5 ± 2.7	314	23.7 ± 3.4	1.3 ± 0.7	43	10.3 ± 5.5	7.7 ± 10.9	1.88 ± 2.0	50
2007	E			27.2 ± 5.8	1.7 ± 0.7	9	6.5 ± 3.8	2.6 ± 6.3		47
	M			25.2 ± 4.8	2.2 ± 0.8	59	15.2 ± 12.6	7.7 ± 10.2		46
	L-1			24.3 ± 4.8	1.5 ± 0.7	38	9.1 ± 6.1	9.0 ± 11.5		49
	L-2			24.0 ± 4.6	2.0 ± 0.9	37	11.2 ± 7.2	7.0 ± 11.9		49

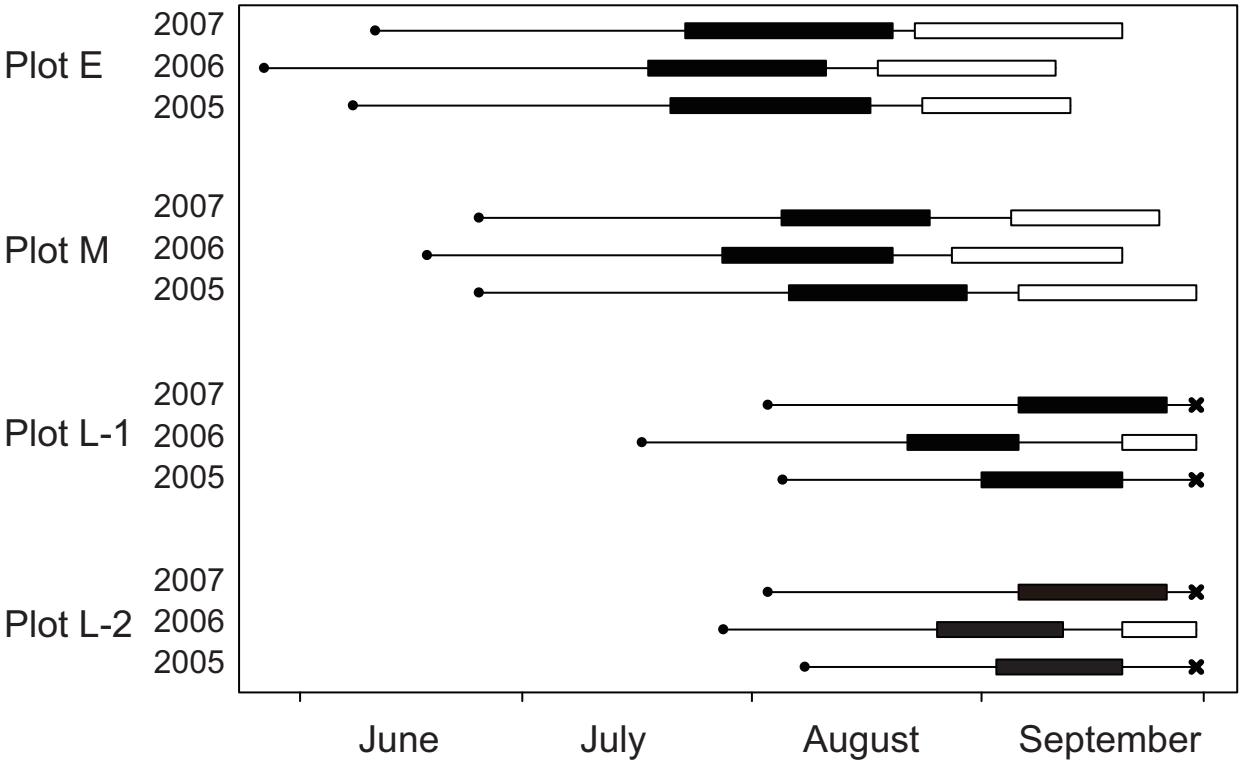
Appendix S6. GLMM result of annual leaf formation. Plot (E, M, L-1, L-2) and leaf number per ramet (ramet size) are included as explanatory variables. Interactions between plot and leaf number were excluded in the best-fitting model.

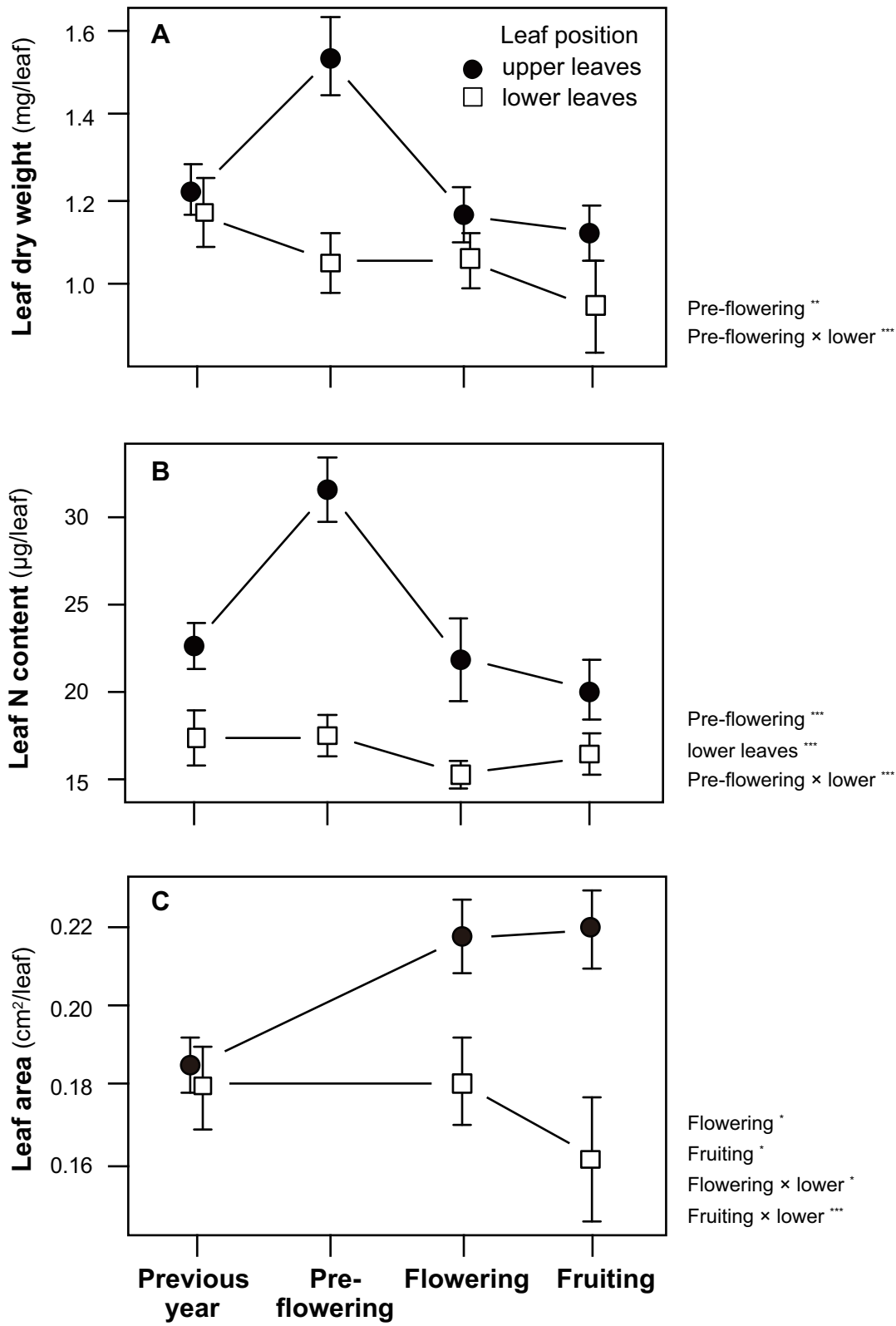
Variable	Coefficient	SE	*z* value	*P* value
Intercept (Plot E)	1.18	0.11	10.83	**<0.001**
Plot M	0.42	0.06	7.54	**<0.001**
Plot L-1	-0.11	0.06	-1.88	**0.059**
Plot L-2	0.03	0.06	0.52	0.60
Leaf number	-0.005	0.002	-2.53	**0.012**

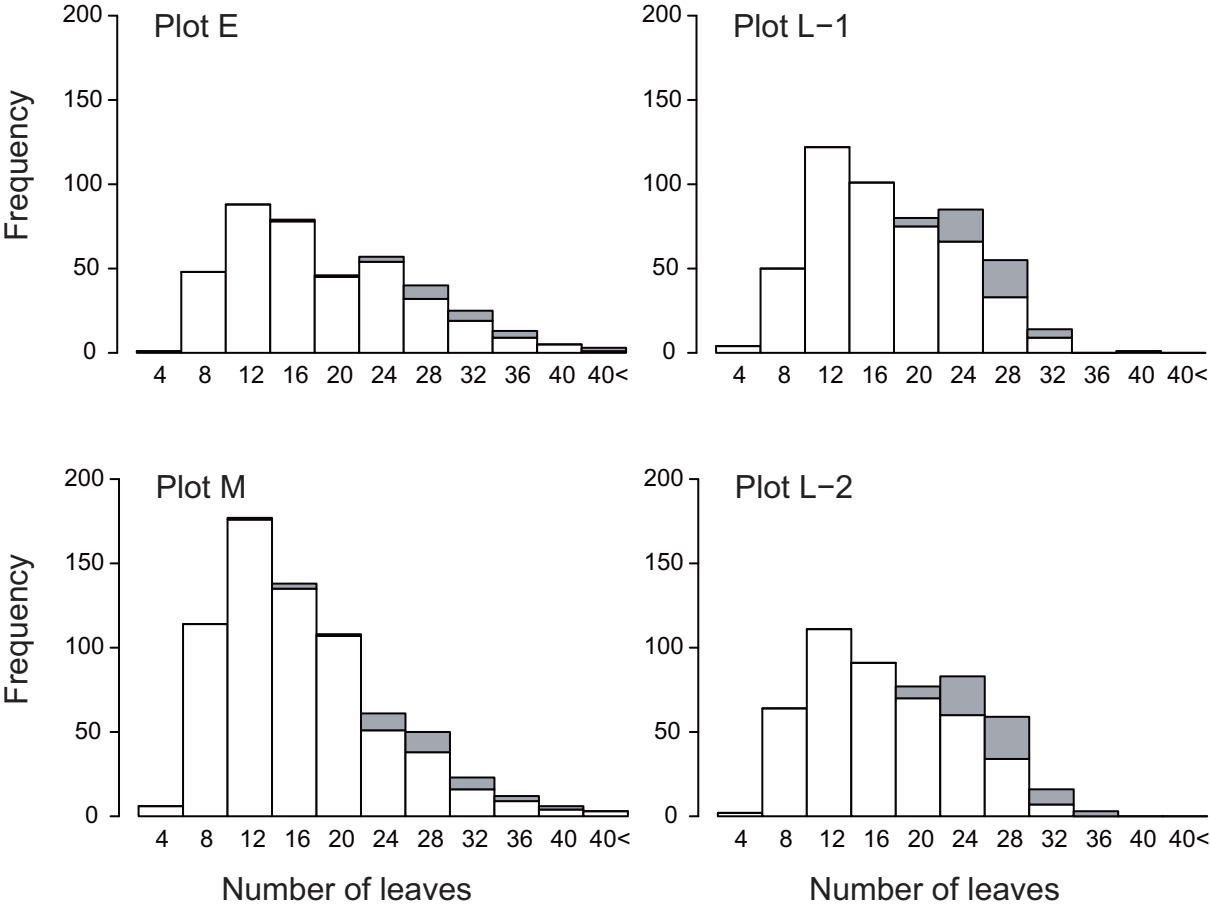
Appendix S7. Cox proportional hazards regression for the survival of vegetative ramets. Plot (E, M, L-1, L-2) and leaf number per ramet (ramet size) are included as explanatory variables. Interactions between plot and leaf number were excluded in the best-fitting model. The sign of coefficient indicates whether each variable is positively or negatively related to the mortality of ramets.

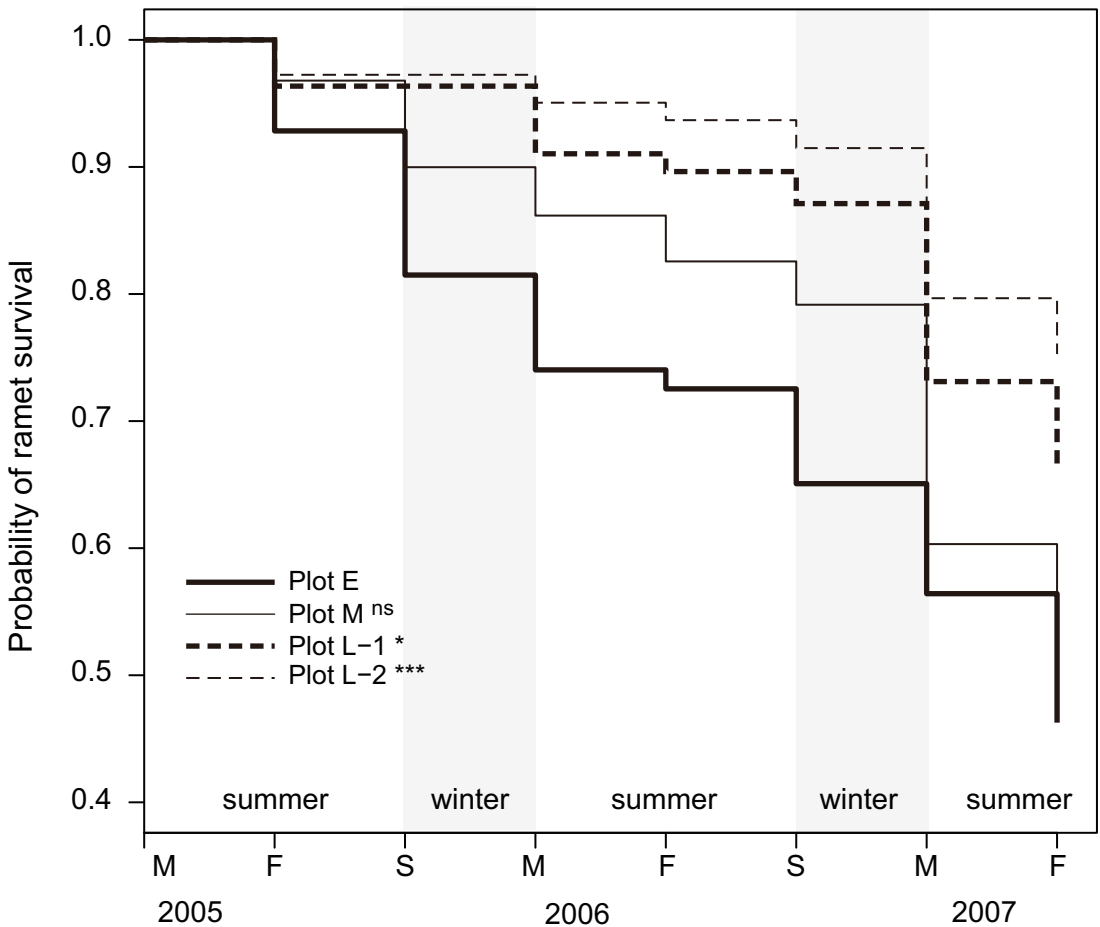
Variable	Coefficient	SE	*z* value	*P* value
Plot E (standard)	0			
Plot M	-0.24	0.23	1.12	0.29
Plot L-1	-0.48	0.24	4.06	**0.044**
Plot L-2	-1.10	0.24	21.41	**< 0.001**
Leaf number	0.02	0.008	8.50	**0.003**

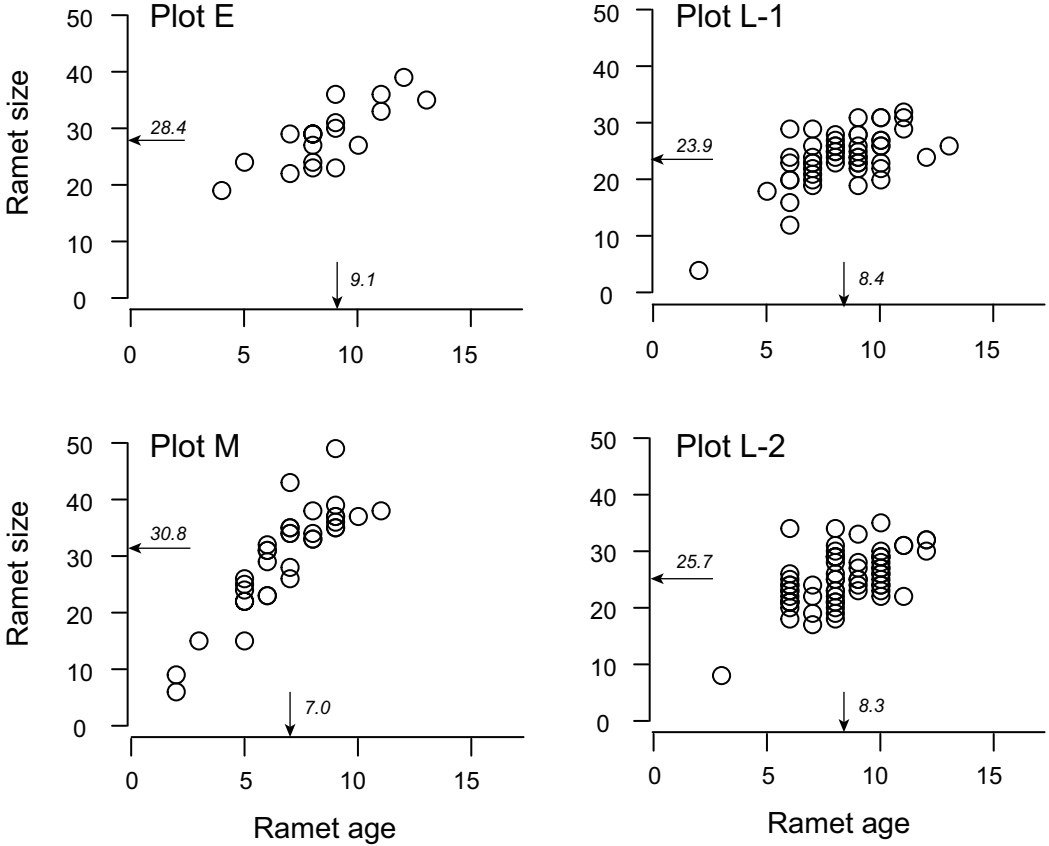
Fig.1 (Kawai & Kudo)

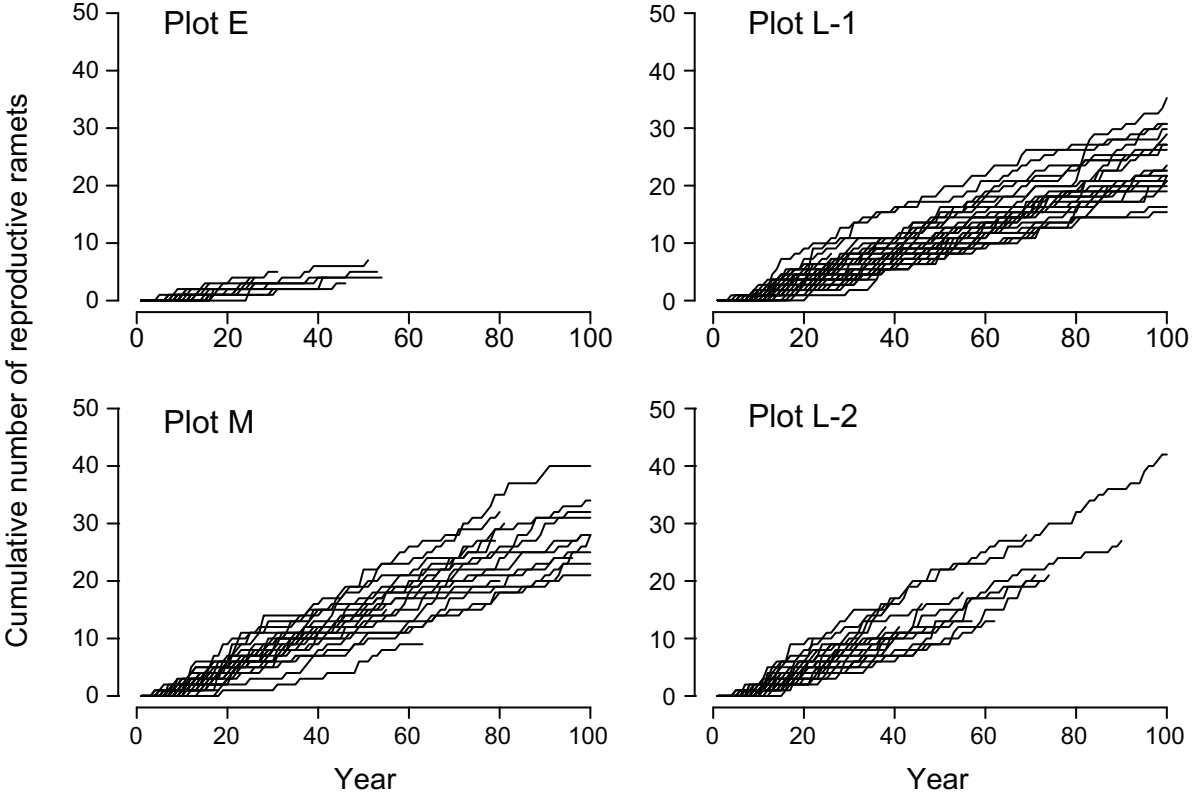



A
Leaf position
upper leaves
lower leaves
Leaf dry weight (mg/leaf)
1.6
1.4
1.2
1.0
Pre-flowering **
Pre-flowering × lower ***
B
Leaf N content (µg/leaf)
30
25
20
15
Pre-flowering ***
lower leaves ***
Pre-flowering × lower ***
C
Leaf area (cm²/leaf)
0.22
0.20
0.18
0.16
Flowering *
Fruiting *
Flowering × lower *
Fruiting × lower ***
Previous year
Pre-flowering
Flowering
Fruiting


Plot E
Plot L−1
Plot M
Plot L−2
Frequency
Frequency
0
50
100
150
200
4
8
12
16
20
24
28
32
36
40
40<
Number of leaves
Number of leaves


Probability of ramet survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
Plot E
Plot M ns
Plot L−1 *
Plot L−2 ***
summer
winter
summer
winter
summer
M
F
S
M
F
S
M
F
2005
2006
2007


Plot E
Plot L-1
Plot M
Plot L-2
Ramet size
Ramet age
28.4
9.1
23.9
8.4
30.8
7.0
25.7
8.3
0
10
20
30
40
50
0
5
10
15


Plot E
Plot L-1
Plot M
Plot L-2
Cumulative number of reproductive ramets
Year
Year
0
10
20
30
40
50
0
20
40
60
80
100