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1 **Original article**

2 **Hares promote seed dispersal and seedling establishment after volcanic eruptions**

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ABSTRACT

Although seed dispersal through animal guts (endozoochory) is a process that determines plant establishment, the behavior of carriers mean that the seeds are not always dispersed to suitable habitats for germination. The germinable seeds of *Gaultheria miqueliana* were stored in the pellets of a hare (*Lepus timidus ainu*) on Mount Koma in northern Japan. To clarify the roles of hares in seed dispersal and germination, field censuses and laboratory experiments were conducted. The field observations were conducted on pellets and seeds in four habitats (bare ground, *Gaultheria miqueliana* shrub patch, *Salix reinii* patch, and *Larix kaempferi* understory), and the laboratory experiments were conducted on seed germination with different light, water potential and cold stratification treatments. The laboratory experiments confirmed that seed germination began a few weeks after the sowing of seeds, independent of cold stratification, when light was sufficient and the water potential was low. The seeds did not germinate at high water potential. The pellets were gradually degraded *in situ*. More seeds germinated from crushed than from intact pellets. Therefore, over the long term, seeds germinated when exposed to light due to the degradation of pellets. The pellets were proportionally dispersed among the four studied habitats. More seeds sown in the field germinated more in shaded habitats, such as in the *Gaultheria* patch and the *Larix* understory, and seeds did not germinate on bare ground, where drought often occurred. Thus, the hares had two roles in the dispersal and germination of seeds: (1) the expansion of *G. miqueliana* populations through seed dispersal to various habitats and (2) the facilitation of delayed seed germination to avoid risks of hazards such as drought. The relationships between small mammals represented by the hare and the shrubs that produce berries are likely to be more mutually evolved than was previously thought.

1. Introduction

Seeds dispersed by animals often determine the spatial structures and dynamics of plant populations (Godinez-Alvarez et al., 2002; Levin et al., 2003). The fates of seeds after dispersal are determined by the suitability of environments for seedling emergence (Schupp et al., 2010). In endozoochory, diaspores transit through the digestive system of animals, and a seed is dispersed to a given habitat, depending on the behavior of the animal (Wenny, 2001). For example, seeds of *Rhus trichocarpa* (Anacardiaceae) pass through the guts of crow to immigrate to bare grounds on the summit of Mount Koma in northern Japan, a suitable habitat for seed germination (Nishi and Tsuyuzaki, 2004). Therefore, understanding the dynamics of plant population requires the identification of the habitats for seed dispersal.

Gaultheria miqueliana Takeda (Ericaceae) forms evergreen shrub patches. The shrubs produce enormous endozoochorous berries during summer and fall on Mount Koma, and the seed banks are found mostly beneath the shrub patches (Uesaka and Tsuyuzaki, 2004). Additionally, mammal pellets are distributed abundantly on the southwestern slope. Preliminary investigations of the pellets confirmed that more than one hundred *G. miqueliana* seeds germinated from the mammalian pellets collected in the early summer of 2001 on Mount Koma (Tsuyuzaki, unpublished data). *Gaultheria miqueliana* seeds require light for germination (Tsuyuzaki & Miyoshi, 2009). Based on the morphological traits of pellets and the fauna of Mount Koma, the pellets were eliminated from the hare, *Lepus timidus ainu* (Leporidae). The common European rabbit, *Oryctolagus cuniculus* (Leporidae), disperses seeds of *Corema album* (Empetraceae) to bare ground (Calvino-Cancela, 2002). However, these mammals are herbivores or frugivores that feed on plants and often reduce seed germinability (Izhaki & Ne'eman, 1997; Chang et al., 2005). Therefore, evaluating the effectiveness of seed dispersal by hares requires assessing the germinability in dispersed habitats.

These results suggested that the feeding on seeds by hares had two effects on the population maintenance and enlargement of *G. miqueliana*: the plant population was enlarged by seed dispersal in hare pellets and was maintained by the development of a seed bank in the pellets in the absence of light. To understand the relationship between seed dispersal by the hare and the germination of *G.*

miqueliana seeds, the following three hypotheses were examined: (1) pellets were distributed evenly across the habitats because hares eliminated at random, (2) seeds germinated in any habitat soon after dispersal when light and moisture were suitable, and (3) the absence of light delayed the germination of seeds embedded in pellets.

2. Materials and methods

2.1. Study sites and plant materials

The study site is located on the southwestern slope of Mount Koma (42°04'N, 140°42'E, 1131 m asl) in southwestern Hokkaido Island, Japan. The climate is warm-cool temperate, and between 1981 and 2010, average annual precipitation was 1097 mm, and the mean annual temperature was 8.1°C (Mori Climatological Observatory, 8 km north from the summit of Mount Koma). Snow generally accumulates from late November to late May. During the survey period, the annual precipitation was 1029 mm and 1278 mm in 2012 and 2013, respectively, and was equivalent to the typical year. However, the rainfall pattern during June and August 2013 was unusual because the total rainfall was only 80 mm, and the daily maximum of 30 mm was recorded during June and July, with a daily average of 1.3 mm. Therefore, the ground surface was dry in non-vegetated areas during this period except after rainfalls. In contrast, total rainfall during August and September was 682 mm, with a maximum of 174 mm and an average of 11.2 mm/day. The total rainfall during October and November was 262 mm.

The mountain is an active, andesitic volcano. The most recent major eruption in 1929 produced 0.38 km³ of ash and 0.14 km³ of volcanic ejecta and induced blast and debris avalanches, which destroyed the former vegetation (Kondo and Tsuyuzaki, 1999). Small-scale eruptions were recorded in 1996, 1998 and 2000, but the effects on vegetation were minimal in the study area (Nishi and Tsuyuzaki, 2004). The recovery of the vegetation was slow, and the amount of bare ground increases with increasing elevation. Four major habitats were recognized for plant colonization (Uesaka and Tsuyuzaki, 2004; Akasaka and Tsuyuzaki, 2009): *Gaultheria miqueliana* patches

(hereafter *Gaultheria* patches), the *Larix kaempferi* understory (*Larix* understory), the *Salix reinii* patches (*Salix* patches) and bare ground. The former three habitats were vegetated with the respective vascular plants, and the bare ground was covered with mosses and lichens, or with nothing. *Gaultheria miqueliana* is an evergreen shrub, *L. kaempferi* a tall tree, and *S. reinii* a deciduous shrub.

Gaultheria miqueliana produces fruits (berries) during early summer and late fall on Mount Koma, and most seeds are dispersed before snowfall. The mature fruits each contain more than 125 seeds, and the seeds are small, approximately 0.065 mg (Tsuyuzaki and Miyoshi, 2009).

2.2. Fate of pellets

To investigate the dynamics of hare pellets, 30 2 m × 1 m plots were established on the southwestern slope at approximately 680 m in early July 2012. The silhouettes of habitats in each plot were sketched on cross-section paper with 10-cm accuracy. The area of each habitat was measured with scan of the silhouettes. All the pellets were removed from the plots before the pellet counts began.

The census was conducted monthly during snow-free periods from August 2012 to November 2013. The census was divided in two phases: the first phase was before June 2013 and the second phase was after July 2013, because the sampling design changed. The locations of newly immigrated pellets were recorded at every census. In the first phase, 42 immigrated pellets were randomly marked and left in the plots to evaluate the natural degradation of the pellets, and 65 pellets were taken to the laboratory. The degradation of pellets in the first phase was evaluated by visually checking each pellet left in the plots until the final census was conducted on November 6, 2013. In the second phase, all immigrated pellets were removed to the laboratory. The seed germination tests of the pellets in the laboratory were performed immediately. The tests were conducted on three-layered filter paper (Whatman #1) in petri dishes at 25°C/5°C with an 18:6 h light:dark cycle.

2.3. Seed germination from pellets in laboratory experiments and on habitats in the field

The temperature and light intensity were measured at 1-hour intervals with three automatic data loggers (HOBO, UA-002-XX, Onset, Bourne) randomly placed on the ground surface in each habitat during the snow-free period from May 23 to November 8, 2013. The photosynthetic photon flux density (PPFD) was calculated from the comparisons between the data loggers and a PAR sensor (S-LIA-M003, Onset). For each habitat, the daily mean temperature and the daily maximum PPFD were calculated.

Seed germination tests were conducted with pellets collected from the southwestern slope between 480 and 900 m asl in early August 2013. The tests were conducted for two months, from late August to late October. Because pellets degraded with time *in situ*, two treatments of pellets were prepared intact and degraded. The degraded pellets were prepared by crushing gently by hands. The germination tests of pellets were conducted in an incubator at 25°C/5°C with an 18:6 h light:dark cycle. In the experiment, one pellet was placed on three layers of filter paper in a petri dish. Each dish was wrapped with Parafilm after 3 ml of distilled water was poured on the filter papers. The seedlings were counted every two weeks until no new seedlings were observed for 10 days. Each treatment included 45 replications.

The fruits of *G. miqueliana* were randomly collected from more than 20 patches in November 2012, and were stored in an incubator at 4°C in the dark. The seeds were extracted from the fruits before sowing. Fifty seeds each were placed in twenty plastic cups (6.5 cm in diameter and 3.5 cm in depth), which were placed in each habitat in June 2013. Each cup was filled with volcanic ash collected from bare ground near the study site. The germinated seeds were counted every two weeks until early November 2013. The viability of the seeds used in the cup experiment was determined using a seed germination test that included eight replicates of 50 seeds in the laboratory at 25°C/5°C with an 18:6 h light:dark cycle.

2.4. Seed germination characteristics

Fruits were collected from more than 20 individuals in early August or early October 2012. The fruits were air-dried in paper bags for a week and were stored in an incubator at 4°C in the dark

until use. The seeds were separated from pulps just before the experiments.

The effects of light, cold stratification and water stress on seed germination were examined. Cold stratification was performed with seeds that were at 4°C in the dark for three time periods, zero, one or five months, on paper moistened with distilled water in petri dishes. To investigate the effects of water stress on seed germination, six levels of water potential, 0, -0.25, -0.5, -1.0, -2.0, -4.0 MPa, were prepared with polyethylene glycol (PEG-6000, Wako, Osaka) (Michel and Kaufmann, 1973). In each dish, 3 ml of solution was poured on the filter paper.

In each petri dish (90 mm diameter), 42 or 50 seeds were sown on the three layers of moistened filter paper, and the dishes were sealed with Parafilm to maintain the moisture. The seed germination tests were conducted at 25°C/5°C without light or with an 18:6 h light:dark cycle. Germination was defined as the emergence of radicles and was determined daily in the light treatment and every four days in the dark treatment. The seed germination tests concluded when germination ceased for more than 10 days, i.e., after 70 days. Eight replications were used for each treatment.

2.5. Statistical analyses

The effects of cold stratification, water potential and light on seed germination rates were analyzed using a generalized linear mixed-effects model (GLMM) with a binomial distribution because the seed germination rates ranged from 0 as the lower limit to 1 as the upper limit (Ronnegard et al., 2010). The total number of germinated seeds was the response variable. The duration (months) of cold, the water potential and the light exposure were the explanatory variables, along with their interactions, and the petri dish code was the random effect. The seeds collected in August and October were analyzed separately.

The daily mean temperature and the daily maximum PPFD were compared between habitats using Tukey's HSD multiple comparison test. The number of pellets was compared among habitats using GLMM with an assumption of a Poisson distribution and with an offset of habitat area and a random effect of plot code. The effect of pellet degradation on the number of germinated seeds was

analyzed by GLMM with a Poisson distribution and a random effect of dish code. The seed germination rates were compared among the four habitats by GLMM with a binomial distribution and a random effect of cup code. All statistical analyses were conducted using the statistical package (ver.2.14.2) (R Foundation for Statistical Computing, Vienna, 2009), which includes the function glmmML in the library glmmML.

3. Results

3.1. Fates of pellets

In total, 148 pellets were recorded in the plots from early August 2012 to early November 2013. The *Salix* patches were the most common, i.e., 21.5 m² (36% of the total plot area), followed by *Larix* understory (20.0 m²; 32%) and bare ground (17.0 m²; 28%) (Fig. 1). The *Gaultheria* patches occupied 2.5 m² (4%). The pellets immigrated to the habitats throughout the survey. By habitat, 10 pellets (7%) were in the *Gaultheria* patch, 20 (13%) in the *Larix* understory, 59 (40%) on the *Salix* patch and 59 (40%) pellets were on the bare ground. The probabilities of numbers of immigrated pellets did not differ significantly among habitats (GLMM, $p > 0.01$), showing that the pellets were distributed equally to all habitats.

In total, 42 pellets that immigrated in the first phase were left in the plots. Of these pellets, by the end of November 2013, 25 were intact, 10 were degraded, and 7 were lost. The first degradation of pellets was observed after overwintering, i.e., in spring of 2013. Of the 42 original pellets, 35 were recovered during the final census in early November 2013 and were tested for seed germination. Seedlings, all of which were *G. miqueliana*, emerged from five of the 35 pellets.

Of the 148 pellets collected after immigration into the plots, 106 pellets were tested for seed germination in the laboratory. Seeds germinated from 22 pellets (21% to total), with an average of 51 seedlings per pellet and a maximum of 129. After the germination tests, a few pellets that did not germinate seeds were investigated under a binocular stereomicroscope, and those pellets did not contain seeds of *G. miqueliana*. Seeds also germinated from pellets collected in June,

demonstrating that seeds in the pellets were viable after overwintering.

3.2. Seed germination by pellet type in the laboratory and in the habitats

Seed germination was observed from 23 of 45 crushed pellets (51%) and from 17 of 45 intact pellets (38%) in the laboratory experiment (Fig. 2). The number of germinated seeds in a pellet was higher in crushed pellets (61 ± 7 , mean with standard deviation; maximum of 253 seeds germinated) than in intact pellets (13 ± 12 , max = 38) (GLMM, $p < 0.01$).

Although seed germination averaged $88\% \pm 7$ in the laboratory, the germination was low in the field, i.e., $22\% \pm 18$ in the *Gaultheria* patch, $17\% \pm 15$ in the *Larix* understory, and $3\% \pm 6$ in the *Salix* patch and zero on the bare ground (Fig. 3). The germination was lower in the *Salix* patch than in the *Gaultheria* patch (GLMM, $p < 0.01$).

3.3. Environmental factors in habitats

Larix kaempferi and *Salix reinii* began leaf flushing in late May and were defoliated in late October. The bare ground had the highest daily maximum PPFD, which was double the PPFD in *Salix* patches (Tukey's HSD test, $p < 0.01$; Fig. 4). The *Gaultheria* patch and the *Larix* understory had the lowest PPFD. The shade in the three vegetated habitats was affected by the foliage phenology and structure of the three species. The canopy of *Salix* was sparse, and light reached the ground surface. In the *Larix* understory, the dense branches and shoots completely covered the ground surface. The solar radiation penetrated the least into *Gaultheria* patches throughout the year because of the dense evergreen foliage. The mean daily temperature was significantly higher on bare ground than in the three vegetated habitats ($p < 0.01$), indicating that shade in the vegetated habitats reduced the temperatures.

3.4. Production and germination of seeds

On the seeds collected in early August, fewer seeds germinated than those collected in early October (Fig. 5). The dark treatment significantly decreased seed germination in all treatments (GLMM, $p < 0.001$; Table 1). Although cold stratification decreased germination of the seeds collected in August, a significant interaction effect indicated that the effects of cold stratification were weakened by high water potential.

The germination of seeds collected in October did not decrease with cold stratification alone but increased with the interaction between light and stratification. The germination of seeds collected in both August and October decreased with increasing water potential from -0.5 to -1.0 MPa, and seeds did not germinate below -2.0 MPa (Fig. 5). The interactions in the GLMMs indicated that water potential altered the effects of light and cold stratification, particularly for the seeds collected in October (Table 1); thus, water potential was crucial for the germination of seeds.

The timing of seed germination was also affected by water potential (Fig. 5). The seeds collected in October began to germinate on the second day, and germination was completed within 30 days when water the potential was lower than -0.5 MPa; however, at -1.0 MPa, complete germination required 56 days. At 0 MPa, the seeds collected in August required 15 days before beginning germination. For seed collected in either season, germination was inhibited and delayed at water potentials below -2.0 MPa. Therefore, the seeds would be in a dormant state with delayed germination when drought occurs.

4. Discussion

4.1. Seed dispersal and germination in habitats

The only seeds in the pellets were from *G. miqueliana*, demonstrating that the hare specialized in dispersal of this species. The Arctic hare (*Lepus arcticus*) has food preferences among Arctic plants and reduces seed viability through long gut passage times (Bruun et al., 2008), and the European hare (*Lepus europaeus*) is less important to local seed dispersal because it reduces seed germinability (Chang et al., 2005). These results suggest that the hares on Mount Koma feed on

large numbers of *G. miqueliana* fruits. The presence of *G. miqueliana* seeds in the pellets demonstrated the food preferences of the hares.

When *G. miqueliana* seeds do not emigrate from the parental patches, most seeds in the fruits are dispersed gravitationally and remain in *Gaultheria* patches. Seeds that accumulate in the patches germinate as a group immediately in the fall, when light and moisture levels are appropriate; further, the seeds that germinate in the autumn often have advantages in seedling survival and growth. For example, the seedlings of *Galium spurium* (Rubiaceae) and *Arabidopsis thaliana* (Brassicaceae) that emerged earlier in the growing season became larger and had better overwinter survival (Masuda and Washitani, 1992; Pico, 2012). For *G. miqueliana*, when the seedling emerged in the fall, seedling growth was insufficient for overwintering. Furthermore, intra-specific competition among seedlings reduced the survival near the parental trees (Packer and Clay, 2000), as occurred in the *Gaultheria* patches when the seeds germinated concurrently. Because seeds in the hare pellets overwintered and had chances to germinate over the long-term during various seasons, the seedlings from pellets avoided intra-specific competition.

The seeds dispersed by hares did not show habitat selectivity, and seeds immigrated to all habitats. By contrast, the seeds dispersed by animals are often disproportionately distributed in specific habitats because of the animals' behaviors. The palm civet disperses *Leea aculeata* (Vitaceae) seeds more in sparse forests because its bird prey favors open habitat (Nakanishi et al., 2010). The scatter-hoarding agouti promotes seed dispersal of a palm, *Astrocaryum standleyanum* (Arecaceae), to well-developed forests because of the high availability of food resources (Hirsch et al., 2012). The common European rabbit, *Oryctolagus cuniculus* (Leporidae), disperses germinable *Corema album* (Empetraceae) seeds mostly to bare ground (Calvino-Cancela, 2002). These observations suggest that the relationships between the *Lepus timidus* and habitat selectivity require further clarification. Furthermore, little is known about the ecology of the hare, likely because of its solitary life style (Dahl and Willebrand, 2005). In a Swedish boreal forest, *Lepus timidus* showed habitat fidelity despite being nonterritorial and occasionally making long excursions from its home range (Dahl, 2005). Such behavior may explain the relationships between the hare and its habitat.

More *G. miqueliana* seedlings emerged from degraded pellets than from intact pellets because

the seeds germinating from the intact pellets were restricted to the surface layer. The pellets degraded gradually, thus delaying the supply of germinable seeds. Long-term or delayed seed germination reduces the risk of seedling extinction in fluctuating and unpredictable environments (Venable and Lawlor, 1980; Rice and Dyer, 2001).

4.2. Seed germination characteristics

The seeds of *G. miqueliana* required light and low water potential for the germination and did not require cold stratification. Therefore, when the moisture was suitable, the seeds germinated soon after exposure to light. However, the rates of seed germination decreased from shaded to exposed habitats, and in particular seeds did not germinate on bare ground which had the highest PPFD. One cause is likely to be the extreme climate in the summer during the surveys, and extreme drought prevented seedling germination even in shaded habitats (Koyama and Tsuyuzaki, 2013). Shade by overstory plants reduced temperature fluctuations and increased moisture in volcanic deposits (Tsuyuzaki et al., 2012), and moisture in volcanic deposits was one of the most important determinants of the timing of seed germination timing of dominant plants on Mount St. Helens, USA (Tsuyuzaki et al., 1997).

4.3. Relationships between hare and *G. miqueliana*

Although the thrush (*Turdus naumanni*) also disperses *G. miqueliana* seeds in its droppings (Nishi and Tsuyuzaki, 2004), most seeds germinate soon after dispersal when habitats are suitable because the fluid droppings are exposed to direct sunlight. The roles of the thrush in seed dispersal are different from the roles of the hare. Furthermore, small seeds, such as *G. miqueliana* seeds, are carried by strong winds, particularly from the bare ground (Akasaka and Tsuyuzaki, 2009). Because the seeds in pellets are immobilized, the seeds stored in pellets provide a supply of seeds to the habitats over the long term. In conclusion, the seeds dispersed by hares had two effects on the population dynamics of *G. miqueliana*: (1) seed were dispersed to various habitats where the parental

patches were not established, and (2) pellets provided the gradual release of a long-term supply of seeds to avoid the risk of concurrent seedling deaths in a fluctuating and disturbed ecosystem, such as on a volcano. *Lepus* hares are widespread in the Northern Hemisphere and have colonized the Southern Hemisphere, where they may alter plant community structure through selective grazing and/or by the dispersal of seeds. For example, *Lepus europaeus* grazed palatable plants and dispersed seeds of both native and exotic species by spreading pellets in Australia (Green and Pickering, 2013). The relationships between small mammalian herbivores including hares and their food plant species may be more specialized than those between large herbivores, such as ungulates, and their plant foods.

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26

Table 1

Coefficients, standard errors (SE) and Z-values of the GLMMs for the effects of light, cold-stratification period and water potential levels on the germination of seeds. The seeds were collected in early August and early October 2012. Crosses (×) indicate interactions. Asterisks (*) indicate significant differences from the control (seeds exposed to light without cold stratification at the water potential of 0 MPa) at $p < 0.001$.

Variable	August			October		
	Coefficient	SE	Z-value	Coefficient	SE	Z-value
Intercept	-0.19	0.16*	-1.15	3.07	0.19*	28.30
Dark	-6.63	1.21*	-5.49	-4.24	0.26*	-32.20
Stratification	-0.56	0.06*	-9.08	0.15	0.07	3.02
Water	3.35	0.35*	9.45	4.83	0.31*	27.24
Dark × stratification	0.28	0.62	0.43	-0.78	0.10*	-10.72
Dark × water	75.93	0.00	0.00	-2.72	0.39*	-14.23
Stratification × water	-0.42	0.10*	-4.32	0.29	0.11*	4.10

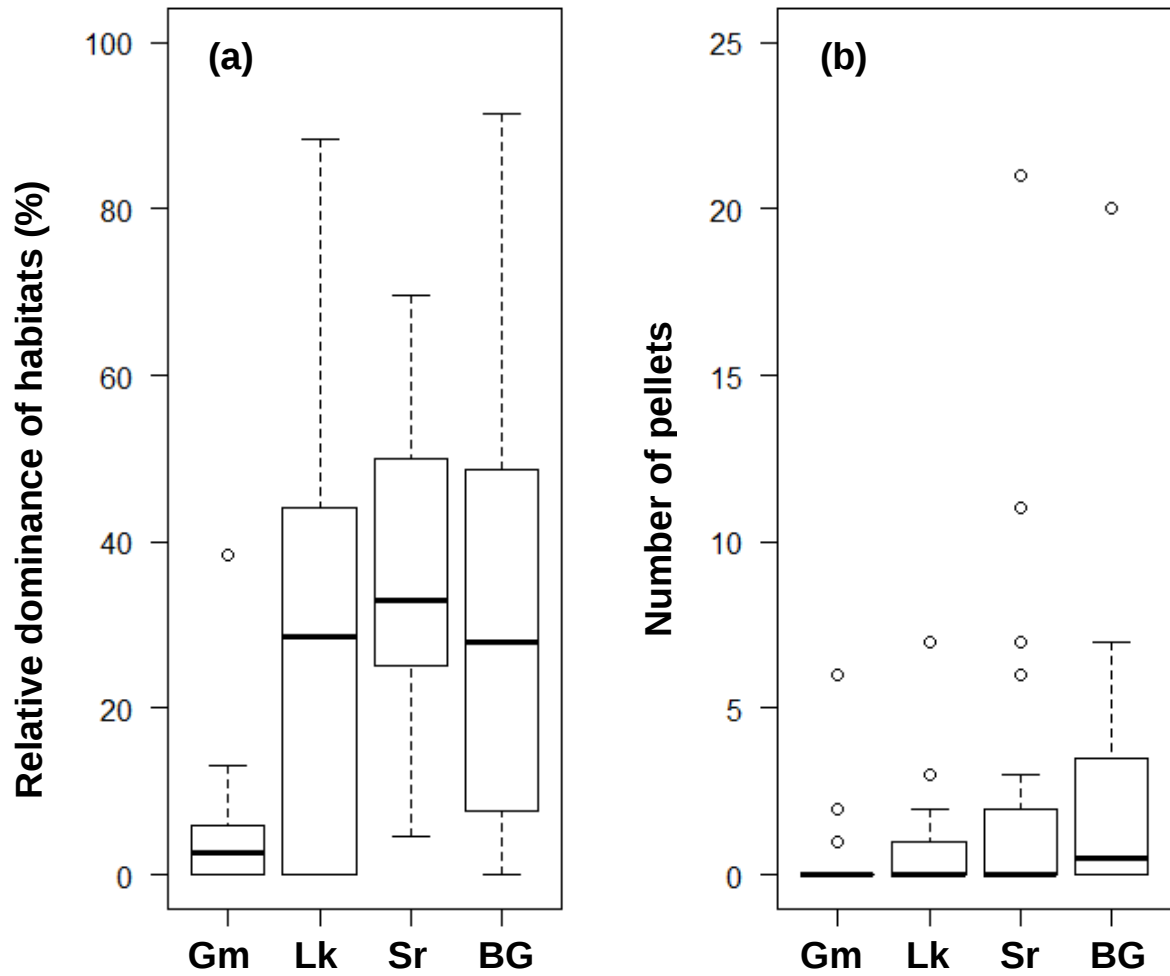
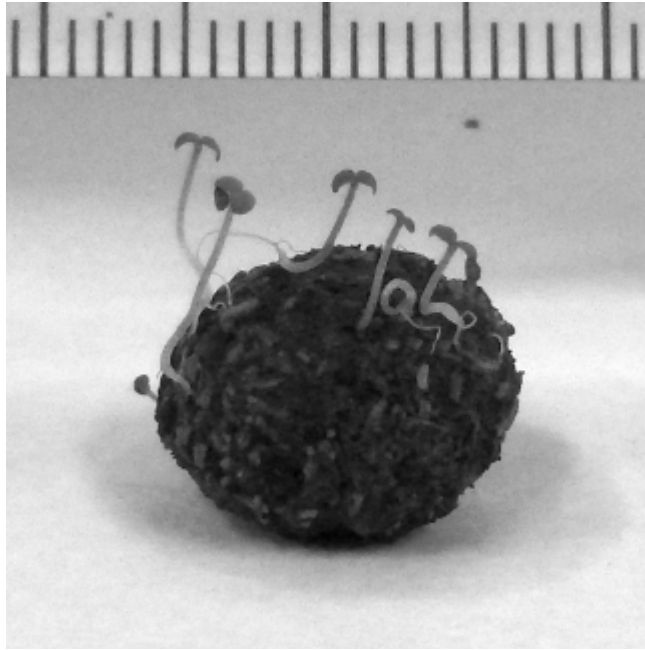


Fig. 1. (a) Relative dominance (%) of four habitats, *Gaultheria* patch (Gm), *Larix* understory (Lk), *Salix* patch (Sr) and bare ground (BG), in each of the 30 2 m × 1 m plots, and (b) number of pellets recorded in the 30 plots during August 2012 and November 2013. The lower and upper margins of boxes represent the 25th and 75th percentiles, respectively. The thick horizontal lines in the boxes represent the medians. The open circles indicate the outliers. The probabilities of numbers of immigrated pellets did not differ significantly among habitats (GLMM, $p > 0.01$).



1 **Fig. 2.** Seeds germinated from an intact pellet one month after laboratory incubation. The pellet
2 was collected from the southwestern slope of Mount Koma in early August 2013. The scale is 1
3 mm.

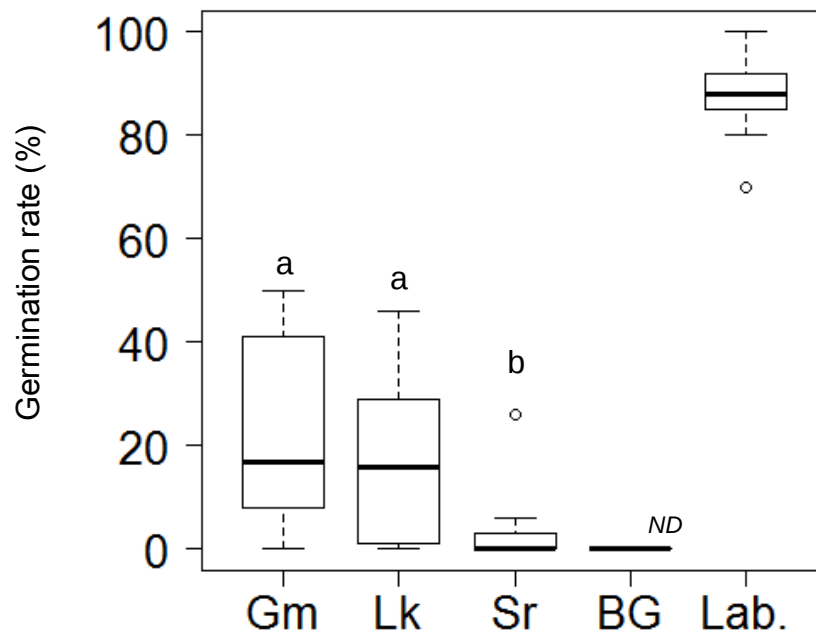


Fig. 3. Germination percentages of *Gaultheria miqueliana* seeds in the four habitats, *Gaultheria* patch (Gm), *Larix* understory (Lk), *Salix* patch (Sr) and bare ground (BG). The germination rate in the laboratory (Lab.) is shown on the right side. The lower and upper margins of boxes represent the 25th and 75th percentiles, respectively. The thick horizontal lines in the boxes represent the medians. The open circles indicate the outliers. Different letters indicate significant differences between the other habitats and the *Gaultheria* patch at $p < 0.01$ (GLMM). ND: not examined because there were too few samples.

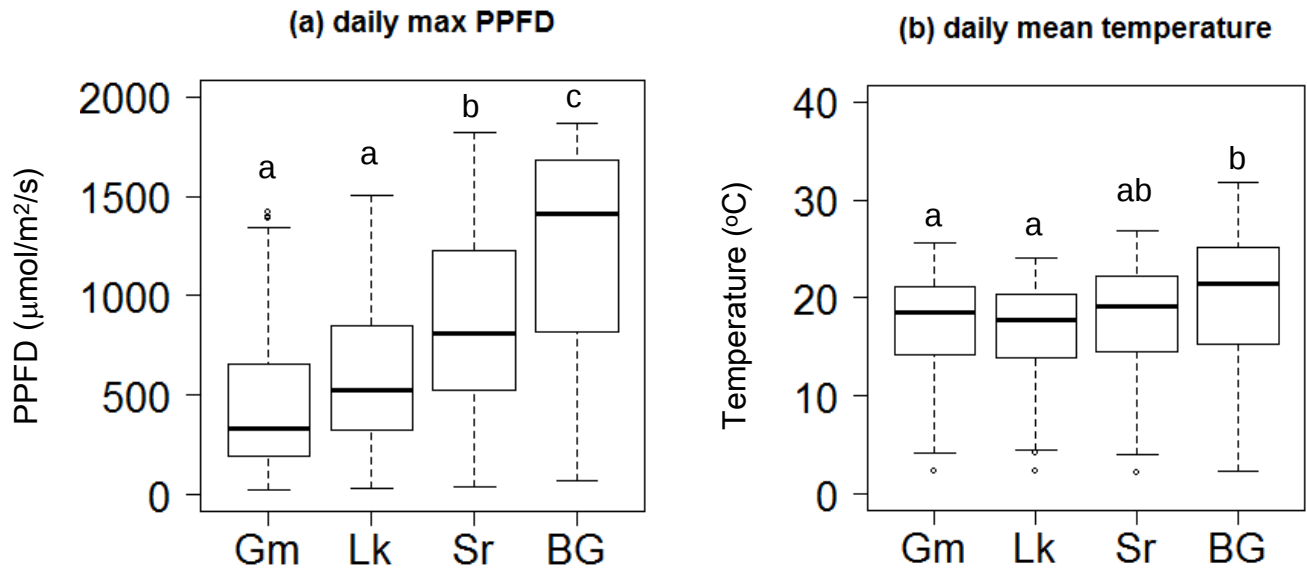


Fig. 4. Daily maximum PPFD (a) and daily mean temperature (b) in the four habitats, *Gaultheria* patch (Gm), *Larix* understory (Lk), *Salix* patch (Sr) and bare ground (BG) from May 22, 2013 to November 7, 2013. The lower and upper margins of the boxes represent the 25th and 75th percentiles, respectively. The thick lines in the boxes represent the medians. The open circles indicate the outliers. Different letters indicate significant differences between the other habitats and the *Gaultheria* patch at $p < 0.01$ (Tukey's HSD test).

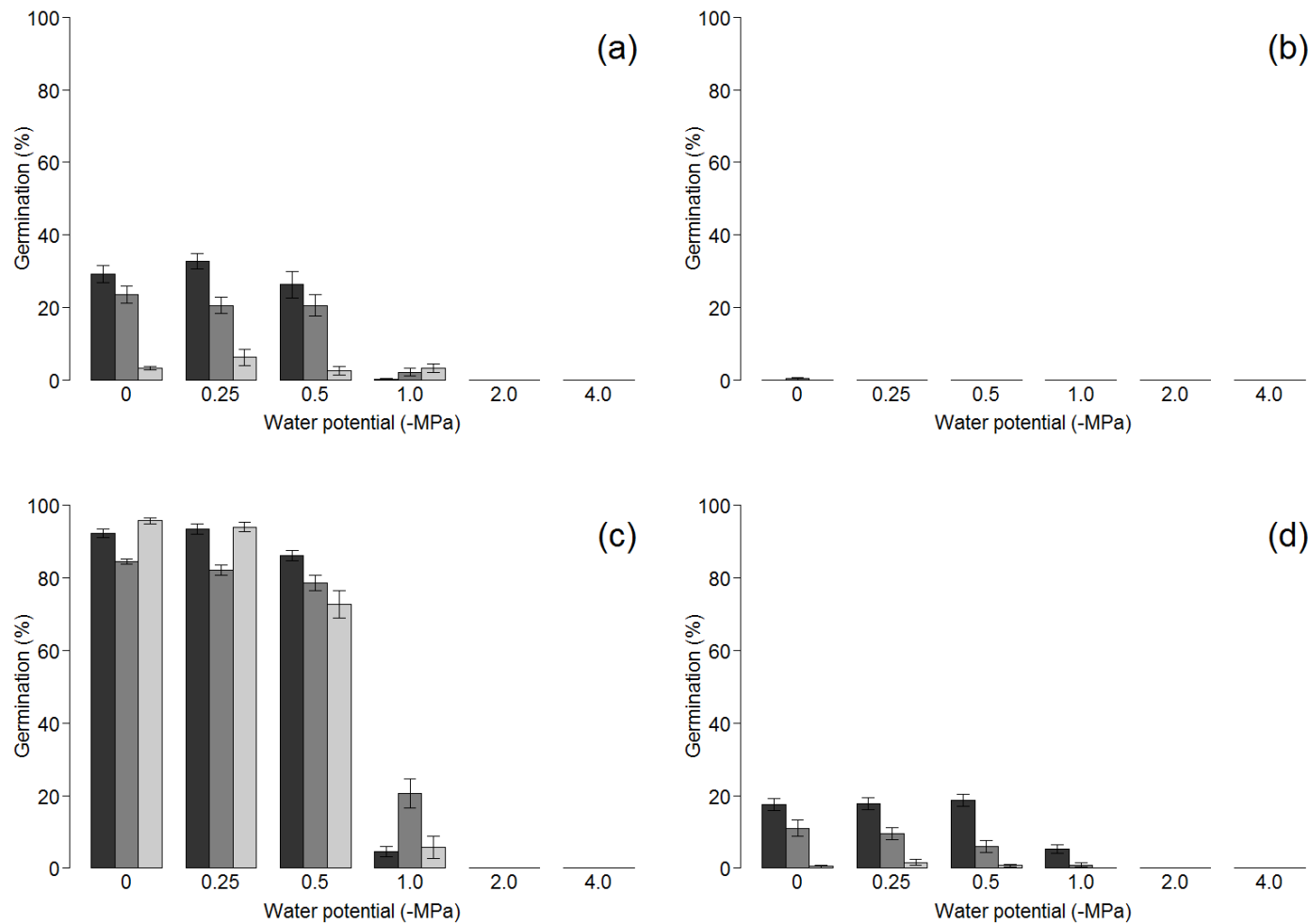


Fig. 5. Germination percentages of *G. miqueliana* seeds after incubation for 70 days. The mean (columns) is shown with standard errors (vertical bars). The treatments are cold stratification for zero (closed columns), one (dark grey) and five months (grey), with water potential at six different levels. The top (a and b) and bottom (c and d) figures are from seeds collected in the early August and early October of 2002 respectively. The left (a and c) and right (b and d) figures show the presence and absence of light, respectively.