

# Multifunctional bracts enhance plant fitness during flowering and seed development in *Rheum nobile* (Polygonaceae), a giant herb endemic to the high Himalayas

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**Abstract** Specialized bracts are thought to be important for the successful reproduction of some plants and are regarded as adaptations to diverse driving forces. However, few empirical studies have quantified the adaptive significance of bracts within a cost–benefit framework. We explored the adaptive significance of large and showy bracts for reproduction in *Rheum nobile*, a giant herb endemic to the high Himalayas. We examined whether the bracts enhance reproductive success during flowering and seed development. Bracts increased flower and fruit temperature on sunny days, greatly decreased the intensity of ultraviolet-B (UV-B) radiation reaching flowers and fruits, and prevented pollen grains being washed away by rain. Experiments indicated that high temperature could promote pollen germination, while pollen grains exposed to rain and

UV-B radiation at ambient levels were seriously damaged. Furthermore, bract removal decreased the number of pollinators visiting flowers. When bracts were removed before or after flowering, fecundity and progeny quality were adversely affected, but seed predation by larvae of pollinators decreased. A cost–benefit analysis demonstrated that the cost of bracts, i.e., increased seed predation, is modest. Our results suggest that the bracts of *R. nobile* promote pollen germination, protect pollen grains from rain and intense UV-B radiation, enhance pollinator visitation during flowering, and facilitate the development of fertilized ovules during seed development. We conclude that multifunctional bracts of *R. nobile* are an effective adaptive strategy in alpine environments and might have been selected for because of abiotic environmental conditions as well as for enhancing pollination success.

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## Introduction

Flowering plants exhibit an astonishing floral diversity to ensure reproductive success (Lloyd and Barrett 1996). Such spectacular variety is largely interpreted as an adaptation to various selection forces, including pollinators, biotic non-pollinators and abiotic factors (Waser and Price 1983; Nilsson 1988; Warren and Mackenzie 2001; Galen and Butchart 2003; Ashman et al. 2004; Strauss and Whittall 2006). Of all the floral traits, bracts are associated with an impressive variety of reproductive strategies. Showy bracts are an effective way to attract pollinators (Armbruster 1997; Herrera 1997; Strauss and Whittall 2006; Sun et al. 2008). For example, in small-flowered

species, showy petaloid bracts help to attract pollinators by enhancing the visual display (Weberling 1992). In addition to pollinator attraction, other biotic factors, for example, protection against nectar robbers and seed predators (Armbruster 1997), may have also played an important role in the evolution of bracts. Furthermore, bracts may provide protection against adverse abiotic factors, such as rain-wash, intense solar radiation, and low temperatures (Sun et al. 2008; Yang and Sun 2009). For example, the large bracts of the dove tree *Davidia involucrata* (Nyssaceae) protect its pollen from rain (Sun et al. 2008). Yang and Sun (2009) found that the bracts of *Saussurea velutina* (Asteraceae) increase the daytime temperature of the species' reproductive organs by 2.5 °C above ambient levels, which may facilitate seed development at high elevations in the Himalayan Hengduan Mountains where this plant grows. Bracts of some plants may be multifunctional, probably responding to a combination of selective forces. For example, the bracts of the dove tree not only protect its pollen from the rain, they also attract pollinators (Sun et al. 2008). Although bracts may represent an important adaptation to abiotic and biotic factors, little attention has been paid to their functional roles and surprisingly few experimental studies have investigated their multifunctionality. Furthermore, floral traits mainly function during flowering, while bracts frequently persist after flowering, suggesting additional functions during seed ripening. To date, however, the functional significance of bracts after flowering has largely been ignored. In addition, although bracts confer benefits, they may also be associated with costs such as herbivore attraction or resource consumption (Keasar et al. 2009; Svensson and Calsbeek 2012). Thus, a complete understanding of the evolutionary significance of bracts in flowering plants requires the consideration of both their benefits and costs.

The high Himalayas are generally characterized by low temperatures, high solar radiation, strong winds, cloudiness, frequent precipitation, and low levels of insect abundance and activity (Ohba 1988; He et al. 2006). Plants in these areas frequently exhibit spectacular floral traits to cope with the hostile environmental conditions (Tsukaya and Tsuge 2001; Körner 2003). *Rheum nobile* Hook.f. and Thomson (Polygonaceae) is a monocarpic perennial herb, endemic to the high Himalayas (Omori and Ohba 1996; Chowdhery and Agrawala 2009). The most outstanding attribute of this species is the large and showy translucent bracts, which cover the towering inflorescences that measure up to 150 cm in height and are visible from far away (Figs. S1a, b). The translucent bracts account for ca. 20 % of aboveground biomass during flowering, but, they have no capacity for photosynthesis because the chloroplasts in them have degenerated (Omori et al. 2000; Zhang et al. 2010a; B. Song et al., unpublished data). The possible role

of the bracts of *R. nobile* has attracted the attention of several authors (Terashima et al. 1993; Omori and Ohba 1996, 1999; Omori et al. 2000; Iwashina et al. 2004; Zhang et al. 2010a). Previous studies have focused on their thermal (Omori and Ohba 1999) and optical characteristics (Omori et al. 2000; Iwashina et al. 2004). These studies found that the presence of the bracts increases the temperature of reproductive organs by 1.5–14 °C over ambient levels and that the bracts could filter out most ultraviolet (UV) radiation (Omori and Ohba 1999; Omori et al. 2000; Iwashina et al. 2004). In addition, previous studies have examined pollen development in response to bract removal; but the specific causes of pollen sterility have not been identified (Omori and Ohba 1996). Furthermore, the bracts of *R. nobile* persist until the seeds have ripened, even after the rosulate leaves have withered (Fig. S1c). However, a possible functional role of the bracts of *R. nobile* during seed development has never been examined. Consequently, to date, the adaptive significance of bracts in *R. nobile* remains obscure.

In this study, we aimed to determine the various functions, during flowering and seed ripening, of the spectacular bracts in *R. nobile*. We postulated that the bracts of *R. nobile* might enhance male fitness by promoting pollen germination, protecting the pollen from damage caused by intense UV-B (280–315 nm) radiation and rain, and attracting pollinators. In addition, we postulated that the bracts continue to have a function after fertilization. Finally, we examined the possible costs of bracts in order to evaluate fully their contribution to reproductive success. The following specific questions were addressed:

1. Is pollen germination sensitive to low temperatures? If so, could bracts increase the temperature of flowers to improve pollen germination?
2. Do intense UV-B radiation and rain damage pollen grains? If so, do the bracts provide protection for the pollen grains?
3. Do bracts enhance pollinator visitation?
4. Does the retention of bracts benefit female fitness during seed development?
5. Do the benefits offset the costs of the bracts?

## Materials and methods

### Study species and sites

*Rheum nobile* (Polygonaceae) is a giant perennial monocarpic herb, occurring at altitudes ranging from 4,000 m to 6,000 m a.s.l., and inhabiting mostly open scree (Omori and Ohba 1996; Chowdhery and Agrawala 2009). Regeneration occurs only from seeds (B. Song et al., unpublished

data). The plant has large rosulate leaves in the vegetative stage and flowers from early June to early July at an age of 5–7 years. The bracts conceal the whole stout conical compound raceme, which consists of 5,000–7,000 flowers growing from a single central axis (Fig. S1a, b, d). The flowers are  $2.31 \pm 0.31$  mm (mean  $\pm$  SE,  $n = 20$ ) wide, have green sepals, no petals and no nectar. Each flower has one ovule and six stamens; single flowers last 3–4 days and the whole inflorescence 7–10 days; *R. nobile* is self-compatible, and autonomous selfing may occur, but the plant depends mostly on insects for pollination (B. Song et al., unpublished data). A species of *Bradysia* (Sciaridae) was the main pollinator. After pollination, these insects deposit their eggs in the ovary and their larvae feed on the mature seed (B. Song et al., unpublished data).

The field experiments were conducted at two sites where the species grows naturally: Huluhai ( $28^{\circ}31'N$ – $99^{\circ}57'E$ , 4,450 m a.s.l.) and Yongjiongzi ( $28^{\circ}24'N$ ,  $99^{\circ}55'E$ , 4,490 m a.s.l.), in Shangri-la County, Yunnan Province, southwest China. Mean annual precipitation (1982–1984) at the nearest meteorological station ( $N28^{\circ}23'$ ,  $E99^{\circ}01'$ , 4,290 m a.s.l.), 95 km from the study sites, was 680–790 mm. The mean annual air temperature is  $-1.0^{\circ}C$  and the difference in mean monthly temperature between the coldest and the warmest month is ca.  $15^{\circ}C$  (Wang 2006). At the two study sites, the vegetation adjacent to the scree includes alpine meadows and *Rhododendron* shrub.

#### Temperature effects on pollen germination

Temperature of the flowers under bracts and in the space between the bracts and flowers was measured using a four-channel thermocouple data logger (Center 309; Center, Taiwan) equipped with four alloy needle-type sensor probes (1–3 mm in diameter and with an active tip length of 5 mm); data were collected between 18 and 23 June 2011. Six flowering plants were selected randomly, and measurements were made at three positions on each plant (top, middle and bottom). On three of the six plants, the bracts were removed with scissors. Air temperature (ca. 70 cm above the ground) was recorded using an integrated thermistor (1,400–104 air temperature sensors; LI-COR, Lincoln, NE). Temperature was recorded at 5-min intervals. The temperature measurements during the seed ripening stage were taken in the same way, but between 10 and 16 August 2011.

We used the method of Patiño and Grace (2002) to determine the effect of temperature on pollen germination. Forty flowers with newly dehiscent anthers were selected randomly from several plants and separated into four groups. Pollen grains from each group were placed into vials containing 10 % sucrose solution and maintained at different constant temperatures (5, 15, 25 and  $30^{\circ}C$ ) in a

water bath; each treatment was replicated 10 times. The lowest value corresponds to temperature of flowers at night, the two middle values to the temperatures of flowers without and with bracts on a sunny day;  $30^{\circ}C$  was selected to test whether higher temperatures inhibited pollen germination. The number of pollen grains that had germinated after 4 h was counted under a light microscope. All the other experiments (i.e., testing for rain and UV-B radiation effects, see below) were conducted at the optimal temperature for pollen germination, as determined in this experiment.

#### UV-B radiation effects on pollen viability

Intensities of UV-B radiation beneath bracts, transitional leaves, and rosulate leaves were measured using UV radiometers (Photoelectric Instrument Factory of Beijing Normal University) on four randomly selected plants at 1400 hours between 12 and 15 June 2011 and between 13 and 16 August 2011 during the plant's flowering and fruiting phases. Similarly, the intensity of UV-B radiation in open air was measured.

To test the response of pollen grains exposed to UV-B radiation, 40 flowers from inflorescences of several plants with newly dehiscent anthers were selected randomly and separated into two groups. The stems of the inflorescences were placed in water to prevent dehydration. The two groups of flowers were exposed to artificial UV-B radiation (302 nm) at an intensity of either  $96.0$  or  $6.5 \mu W cm^{-2} s^{-1}$  provided by an UV analyzer (Shanghai Science and Analytical Instruments, Ji-hui, Shanghai); these values correspond to the intensity of UV-B radiation either in the open air or beneath the bracts. After exposure to UV-B radiation for 2 or 4 h, pollen grains from the ten replicates of each treatment were placed in sucrose solution at a concentration of 10 % by mass. For each replicate, the number of pollen grains that had germinated after 4 h was counted under a light microscope.

#### Effect of rain on pollen grains

We randomly selected eight flowering plants from the Huluhai population and 20 flower buds in total were marked. Nylon mesh bags were used to exclude insects; the marked flowers were divided into two groups once the anthers of selected buds began to shed pollen. In the first group, the bracts were removed just before rainfall; in the second group the bracts were left intact as a control. After rainfall, all the anthers of each selected flower in the two groups were collected and fixed in FAA solution to allow the pollen grains to be counted.

To test the effect of rainfall on pollen viability, we used Dafni's (1992) method to assess pollen germination.

Twenty flowers with newly dehiscid anthers were selected randomly and separated into two groups. In the first group, pollen grains were placed in sucrose solution at a concentration of 10 % by mass, reportedly the optimum concentration for pollen germination in many plant species (Huang et al. 2002; Sun et al. 2008; Wang et al. 2010). The second group of pollen grains was placed in distilled water (0 % sucrose solution). Each treatment was replicated 10 times. In each treatment, the number of pollen grains that had germinated after 4 h was counted under a light microscope.

#### Pollinator visitation

To test whether bracts enhance pollinator visitation, eight flowering plants were selected randomly each day between 18 and 21 June 2011 and separated into two groups. The bracts of the first group were removed, while the rest were left intact as a control. The numbers of day-time pollinators were recorded at 0900, 1200, 1500 and 1800 hours. Nocturnal pollinator observations were carried out at 2100 hours using a flashlight. During each census, we uncovered the bracts to record the number of fly fungus gnats on the flowers, taking care not to disturb the pollinators.

#### Effects of bracts on fecundity and progeny quality

To test the effect of bracts on fecundity and progeny quality, 24 flowering plants were selected randomly from both the Huluhai and Yongjiongyi populations. In each population, the selected plants were randomly assigned to the following groups:

1. Bracts removed before flowering: bracts were carefully folded and held in place by thin wires (Fig. S1e) to expose all the flowers before flowering, effectively removing any influence of the bracts. We then randomly selected seven flower heads on each plant and marked them.
2. Bracts removed after flowering: when all flowers on a plant had been pollinated, seven flower heads on each plant were randomly selected and marked, and the bract treatment was performed as described for the first group.
3. Control: plants were left intact and seven flower heads on each plant were marked randomly.

When fruits were ripe (in early September), all marked flower heads were collected and taken to the laboratory to determine fruit set, seed abortion rate and seed predation rate; each flower head was treated as a single unit. We observed that larvae of *Bradysia* sp. developing within the fruit chew through the outer coat once the seed is eaten;

they then fall to the ground where further development occurs. Seed predation was determined by recording the number of fruits with hollowed-out fruit coats. In the Huluhai population, one plant in the first group and one control plant had been destroyed and could not be included in the results. For each treatment in each population, the mass of ten seeds was determined; this measurement was replicated 25 times. In addition, the seed germination rate for each of the three groups was determined. Six replicates of 25 seeds from each group were placed on wet filter paper in petri dishes. The seeds were maintained at 15 °C in an incubator with 12 h day<sup>-1</sup> of white light from cool-white fluorescent lamps. The number of germinated seeds in each petri dish was recorded 30 days after sowing.

#### Benefits and costs of bracts

We considered decreased seed abortion and increased fruit set and seed germination to be benefits, whilst higher levels of seed predation by *Bradysia* sp. was a cost associated with the presence of bracts. To quantify the benefits and costs of bracts with respect to reproduction in *R. nobile*, we compared the fruit set, seed abortion, seed predation and seed germination from different treatments. In addition, we also compared the cumulative probability (CP) of reproduction from pollination to seed germination (Gulias et al. 2004), which represents the overall reproductive success. To determine the CP value, four transition probabilities (TPs) between different stages were calculated as follows: TP1 was equivalent to fruit set, i.e., the number of fertilized flowers (with expanded ovules) in relation to flowers produced; TP2 was the probability of a seed reaching maturity, which was equal to 1 minus the abortion rate; TP3 was the proportion of viable seeds, i.e., the probability of a seed escaping predation by *Bradysia* sp. larvae, which was equal to 1 minus the predation rate; TP4 was the probability of a seed germinating successfully. Thus, CP was equal to  $TP1 \times TP2 \times TP3 \times TP4$ .

#### Data analysis

In this study, with the exception of seed mass and seed germination, the experimental units were either flowers or plants and, accordingly, prior to statistical analysis we calculated means for either flowers or plants from the measurements. One-way ANOVA was used to test the effect of temperature on the germination of pollen grains, and the effects of bracts on the intensity of UV-B radiation, fecundity and progeny quality, respectively. In order to test the effects of the intensity of UV-B radiation and time on germination of pollen grains, and of bracts and time on pollinator visitation, two-way ANOVA was used. In addition, the effects of rain on the number of remaining pollen

grains and on the germination of pollen grains were examined using independent-sample *t*-tests. Multiple comparisons of means were performed by Duncan's test at the 0.05 significance level. Data were  $\log_{10}$  transformed or square root-arc sine transformed if necessary. All analyses were performed using SPSS version 18.0.

## Results

### Temperature effects on pollen germination

During flowering and seed development, the temperatures of flowers or ripening fruits concealed by bracts and in the spaces between bracts and flowers (or fruits) were higher by up to 10 and 8 °C, respectively, than when bracts were removed or in the ambient conditions on sunny days (Fig. 1a, b). However, rain and cloud decreased such temperature differences, and nighttime temperatures differed little between treatments (Fig. 1a, b).

There was a significant effect of temperature on the germination of pollen grains ( $F_{3, 36} = 190.30$ ,  $P < 0.001$ ; Fig. 2a). When pollen grains were maintained at 25 °C, germination was  $51.82 \pm 2.64$  %, while only  $4.18 \pm 0.42$  % and  $17.50 \pm 1.35$  % of pollen grains germinated at 5 and 15 °C, respectively. However, germination of pollen at 30 °C did not differ significantly from that at 25 °C (Fig. 2a).

### UV-B radiation effects on pollen viability

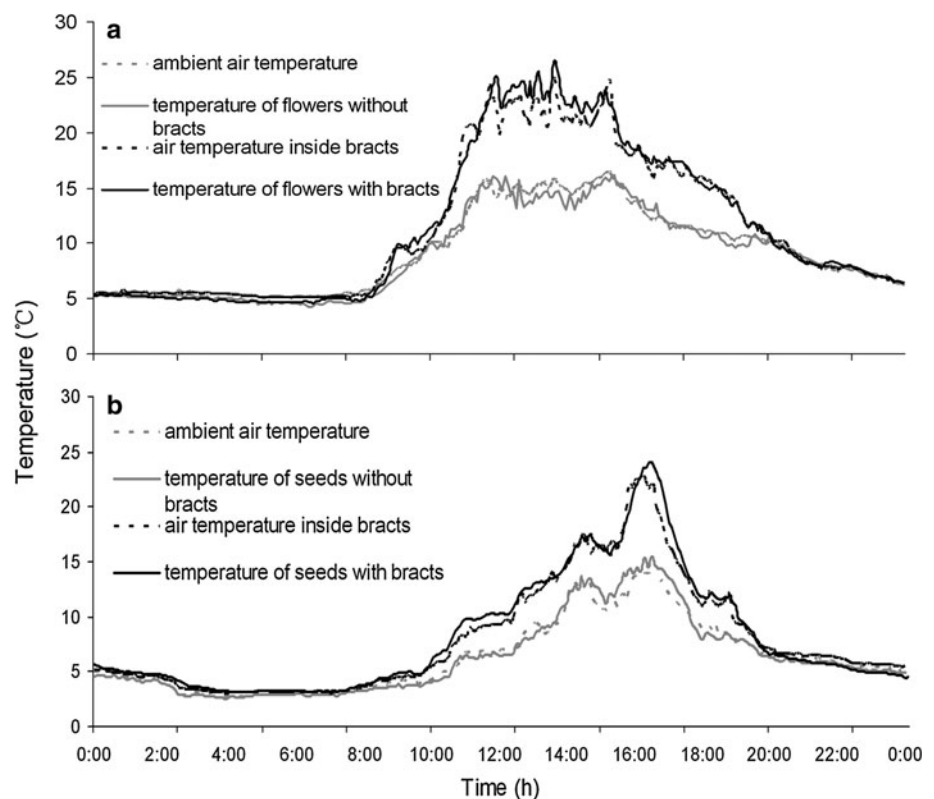
The intensity of UV-B radiation beneath bracts was significantly lower than in the open air, but higher than beneath transitional and rosulate leaves during both flowering and fruiting (flowering,  $F_{3, 60} = 33207.06$ ,  $P < 0.001$ ; fruiting,  $F_{3, 60} = 28072.57$ ,  $P < 0.001$ ; Fig. 3). The intensity of UV-B radiation reaching flowers (or fruits) was decreased by 93–98 % by bracts.

The germination of pollen grains exposed to UV-B radiation of  $96 \mu\text{W cm}^{-2} \text{ s}^{-1}$  was lower than that of pollen grains exposed to  $6.5 \mu\text{W cm}^{-2} \text{ s}^{-1}$  ( $F_{1, 1} = 764.03$ ,  $P < 0.001$ ; Fig. 2b). In addition, the germination of pollen grains exposed to UV-B radiation for 4 h was significantly lower than for those exposed for 2 h ( $F_{1, 1} = 15.05$ ,  $P < 0.001$ ; Fig. 2b). However, germination of pollen grains was not affected by the interaction between treatment and time ( $F_{1, 36} = 1.78$ ,  $P = 0.19$ ; Fig. 2b).

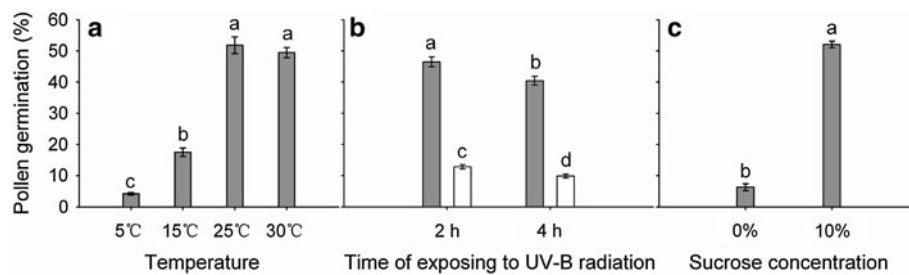
### Effect of rain on pollen grains

When bracts were removed, most of the pollen grains were washed away, unlike in the control (Fig. S1f, g). After rainfall, the number of remaining pollen grains from flowers without bracts was significantly lower ( $212 \pm 341$ ) than from flowers with bracts ( $5596 \pm 1066$ ,  $t = -15.20$ ,  $df = 18$ ,  $P < 0.001$ ).

**Fig. 1** Variation in temperature in four different treatments during flowering (**a** rain started at 1515 hours on 19 June 2011) and fruiting (**b** rain started at 1630 hours on 17 August 2011) ( $n = 3$ )

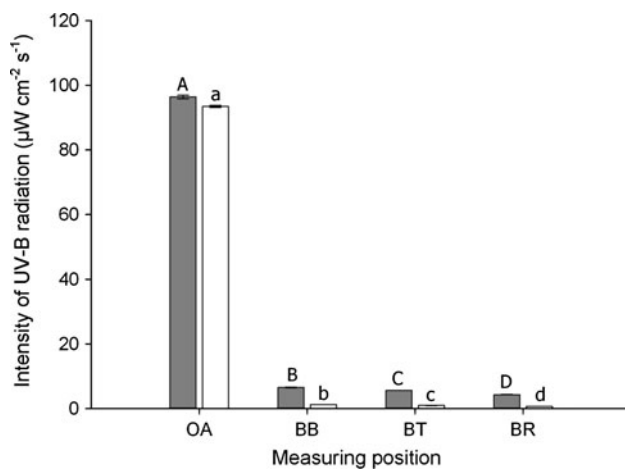




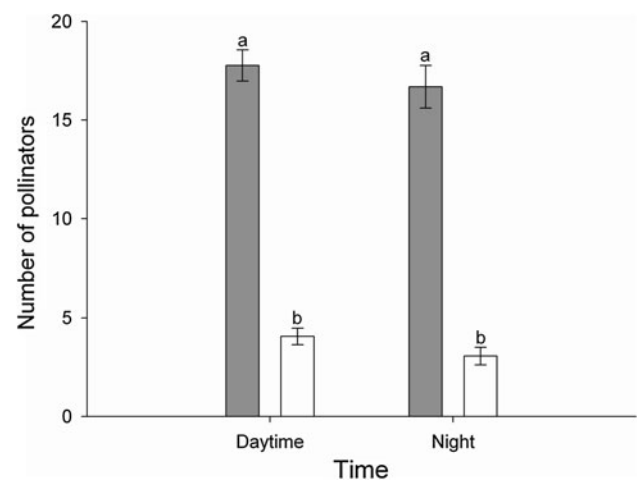


**Fig. 2** Pollen germination (%; mean  $\pm$  SE) of *Rheum nobile*. **a** In 10 % sucrose solution at different temperatures ( $n = 10$ ); **b** in 10 % sucrose solution after 2 h and 4 h exposure to different intensities of ultraviolet-B (UV-B) radiation: 6.5  $\mu\text{W cm}^{-2} \text{s}^{-1}$  (dark gray bars),

96.0  $\mu\text{W cm}^{-2} \text{s}^{-1}$  (white bars) ( $n = 10$ ); **c** in 0 % (distilled water) and 10 % sucrose solution ( $n = 10$ ). Different letters denote significant differences at  $P < 0.05$



**Fig. 3** Intensity of UV-B radiation (mean  $\pm$  SE) in open air (OA), beneath bracts (BB), beneath transitional leaves (BT) and beneath rosulate leaves (BR) during flowering (dark gray bars) and fruiting (white bars) ( $n = 16$ ). Different letters denote significant differences at  $P < 0.05$



**Fig. 4** Number of pollinators (mean  $\pm$  SE) on each flowering *R. nobile* plant with bracts (dark gray bars) and without bracts (white bars) during the day and at night, respectively ( $n = 16$ ). Different letters denote significant differences at  $P < 0.05$

The germination of pollen grains in 10 % sucrose solution was significantly higher ( $52.07 \pm 1.03$  %) than in 0 % sucrose solution ( $6.31 \pm 1.11$  %,  $t = 30.11$ ,  $df = 18$ ,  $P < 0.001$ ; Fig. 2c).

#### Pollinator visitation

Fly fungus gnats (*Bradysia* sp.) preferred to visit plants with bracts ( $17.42 \pm 1.00$  individuals on each plant) over those without bracts ( $3.92 \pm 0.46$  individuals on each plant;  $F_{1, 1} = 336.29$ ,  $P < 0.001$ ; Fig. 4). However, there was no difference in the number of pollinators on flowers when day and night were compared ( $F_{1, 1} = 0.50$ ,  $P = 0.48$ ); similarly, there was no significant interaction between treatment and time ( $F_{1, 60} = 0.35$ ,  $P = 0.56$ ).

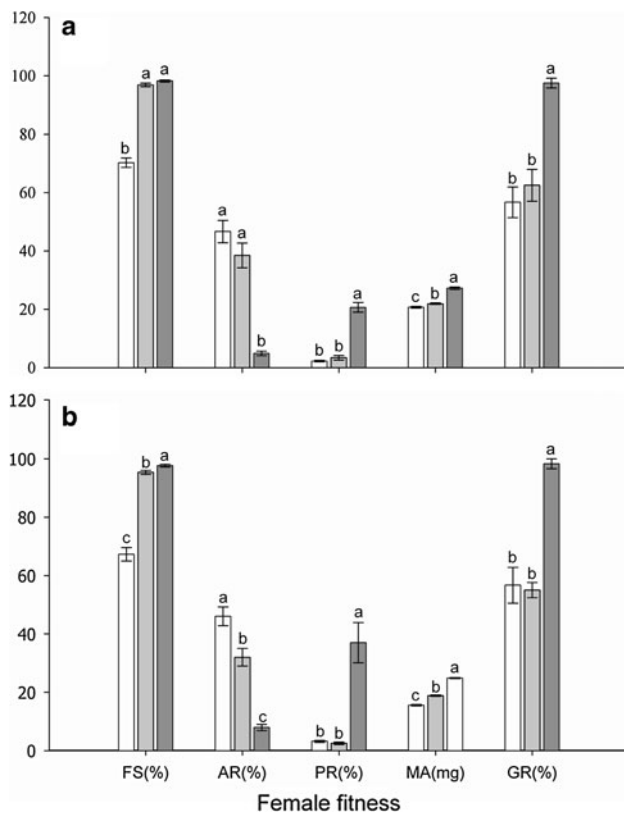
#### Fecundity and progeny quality

In the Huluhai population, fruit set of the control plants was significantly higher than that for plants with their

bracts removed before flowering, but not higher than those that had their bracts removed after flowering ( $F_{2, 19} = 219.70$ ,  $P < 0.001$ ; Fig. 5a). In the Yongjiongqi population, fruit set in the control plants was significantly higher than in plants with their bracts removed before or after flowering ( $F_{2, 21} = 146.63$ ,  $P < 0.001$ ; Fig. 5b). In both populations, seed abortion in control plants was significantly lower than in plants with their bracts removed before or after flowering (Huluhai,  $F_{2, 19} = 42.25.70$ ,  $P < 0.001$ ; Yongjiongqi,  $F_{2, 21} = 53.89$ ,  $P < 0.001$ ; Fig. 5a, b).

However, seed predation in the control plants was significantly higher than in plants with their bracts removed before or after flowering in both the Huluhai ( $F_{2, 19} = 101.05$ ,  $P < 0.001$ ; Fig. 5a) and the Yongjiongqi ( $F_{2, 21} = 24.30$ ,  $P < 0.001$ ; Fig. 5b) populations.

Seed mass was higher in the control plants than in those with their bracts removed before or after flowering (Huluhai,  $F_{2, 72} = 109.17$ ,  $P < 0.001$ ; Yongjiongqi,  $F_{2, 72} = 408.67$ ,  $P < 0.001$ ; Fig. 5a, b).



**Fig. 5** Comparisons of female fitness (mean  $\pm$  SE) among plants with bracts removed before flowering (white bars), plants with bracts removed after flowering (light gray bars), and control plants (dark gray bars), at Huluhai (a) and Yongjiongnyi (b). Different letters denote significant differences at  $P < 0.05$ . FS Fruit set, AR seed abortion rate, PR seed predation rate, MA seed mass, GR seed germination rate

Seed germination for the control plants was significantly higher than for plants with their bracts removed before or after flowering in both populations (Huluhai,  $F_{2,15} = 24.27$ ,  $P < 0.001$ ; Yongjiongnyi,  $F_{2,15} = 38.29$ ,  $P < 0.001$ ; Fig. 5a, b).

#### Benefits and costs of bracts

Bracts increased reproductive success by increasing pollination success, decreasing seed abortion, and enhancing germination success (Fig. 6). Fruit set was increased by 27.5 % as a result of bracts being present during flowering. Seed abortion was reduced by 29.5 and 40 % as a result of bracts being present during flowering and fruiting, respectively. Seed germination was increased by 39 and 41 % as a result of bracts being present during flowering and fruiting, respectively. However, the presence of bracts had an associated cost in terms of increased seed predation by *Bradysia* sp. Compared with plants with bracts removed before or after flowering, seed predation of plants with

intact bracts was 26 and 26.5 % higher, respectively. In summary, the cumulative reproductive success in the presence of bracts (63.7 %) exceeded that when bracts were removed either before (20.2 %) or after (35.5 %) flowering.

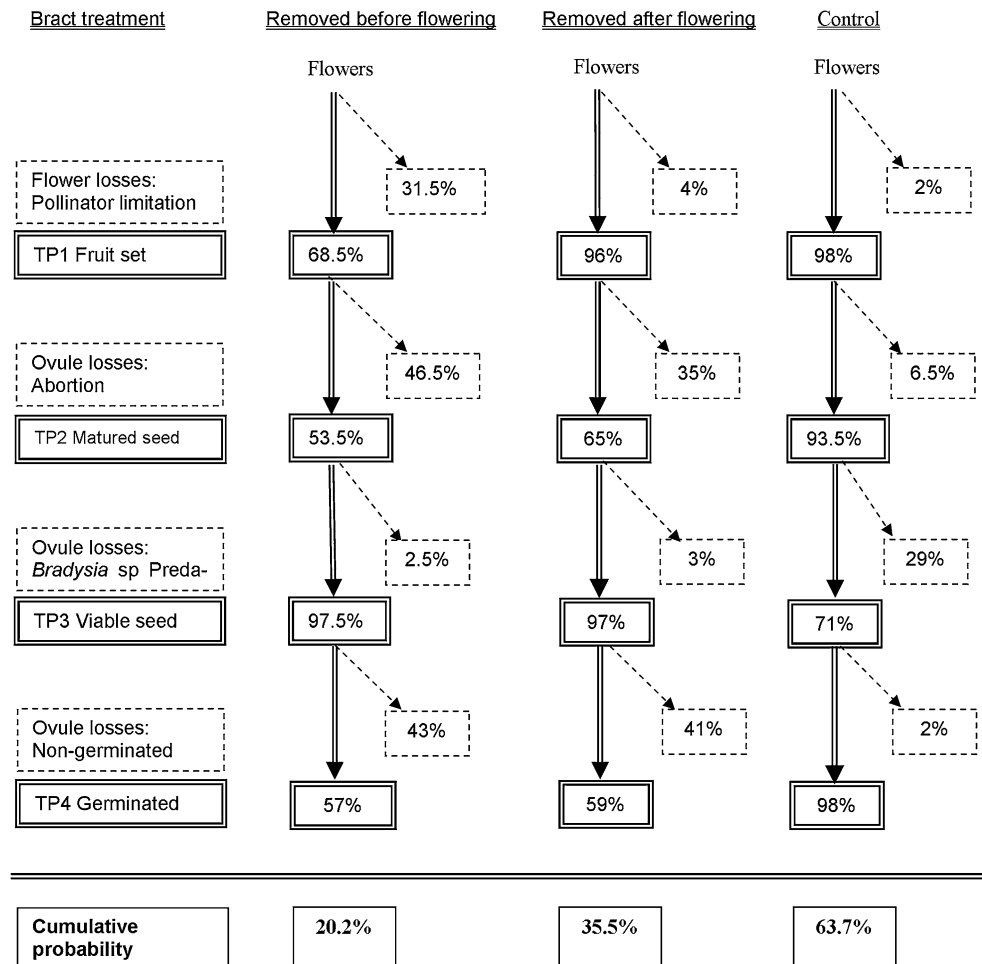
#### Discussion

This study examined, experimentally, the various functions of bracts in *R. nobile*. Unlike previous studies on bract function, which have mainly focused on pollinator attraction during flowering (Herrera 1997; von Balthazar and Endress 1999; Borges et al. 2003; Keasar et al. 2009), our study shows that the bracts of *R. nobile* are functionally important not only because they enhance pollen germination, protect pollen grains and attract pollinators during flowering, but also because they facilitate the development of fertilized ovules during fruiting. Accordingly, the bracts of *R. nobile* enhance male and female fitness during and after flowering. Our cost–benefit analysis indicated that the presence of bracts has a net benefit. In conclusion, our results suggest that the bracts of *R. nobile* are a multi-functional adaptation, enhancing reproductive success in harsh alpine environments.

#### Bracts promote pollen germination and protect pollen grains

The temperature of flowers concealed by bracts was up to 10 °C higher than those without bracts on a sunny day. A similar effect has also been reported in *R. nobile* in the Nepal Mountains (Omori and Ohba 1999) and *Saussurea velutina* in the same region (Yang and Sun 2009). Unlike the results reported by Omori and Ohba (1999), there was no difference in nighttime temperature between flowers concealed by bracts and ambient air. The temperature increase associated with the presence of bracts was only apparent on sunny days probably because heat production resulted from high transmittance of photosynthetically active and infrared radiation by the bracts (Omori et al. 2000) rather than because of active metabolism, for example, cyanide-insensitive respiration (Skubatz et al. 1990; Seymour et al. 2003). The pollen grains of many plants still remain viable at temperatures below zero (Hughes and Lee 1991; Lora et al. 2006). Thus, it could be inferred that low temperatures at night and on rainy days have no adverse effect on the viability of pollen in *R. nobile*. However, temperature has been considered to be a critical factor for pollen germination on the stigma after pollination (Chakrabarti et al. 2011). Our pollen germination experiment showed that pollen germination in *R. nobile* was highly sensitive to temperature, with significantly

**Fig. 6** Flow chart of reproductive gains (*solid lines*) and losses (*dotted lines*) from flower to seed germination for *R. nobile* when bracts were either removed before or after flowering and compared to control plants. *TP* Transition probability



higher germination rates at 25 °C than at 15 °C; these were, respectively, the highest temperatures of flowers with and without bracts on the sunny day when data were collected (Fig. 2a). Therefore, like the heliotropism of flowers of *Ranunculus adoneus* (Galen and Stanton 2003), the heating resulting from the presence of bracts in *R. nobile* could help pollen to germinate faster on the stigma and facilitate fertilization. Although overheating can inhibit pollen germination (Sato et al. 2002; DeCeault and Polito 2010; Zhang et al. 2010b), we found no evidence of heat adversely affecting pollen germination in *R. nobile*, because the temperature of flowers covered by bracts never exceeded 30 °C during our measurements.

Intense UV-B radiation reduced pollen germination significantly, and, the longer the pollen grains were exposed to UV-B radiation, the lower the germination rate (Fig. 2b), indicating that the viability of pollen in *R. nobile* is susceptible to intense UV-B radiation, as reported in other studies (Feng et al. 2000; Wang et al. 2010). Flavonoids in the bracts of *R. nobile* are able to absorb almost all the UV radiation (Omori et al. 2000; Iwashina et al. 2004),

thus the intensity of UV-B radiation reaching flowers concealed by bracts was found to be only 6.8 % of the ambient level. Several alpine plant species have mechanisms to avoid UV-B damage, including pubescence and nodding flowers (Caldwell 1981; Yang and Sun 2009; Wang et al. 2010). For example, *Anisodus luridus* growing on the Qinghai-Tibet Plateau protects its pollen grains from intense UV radiation by the flowers nodding during flowering (Wang et al. 2010). Torabinejad et al. (1998) suggested that the earlier in the year that plant species flower, the more likely they are to be susceptible to UV-B radiation. Flowering of *R. nobile* occurred in June, i.e., in early summer when the UV-B radiation intensity tends to be at its highest in this region (Zhu et al. 2005). Obviously, the bracts of *R. nobile* also function as anti-UV “parasols” thus decreasing UV-B radiation damage.

The flowering of *R. nobile* generally started in early June and continued until early July, which coincides with the rainy season in the eastern Himalayas. According to records from the nearest meteorological station, total precipitation amounts to 125 mm in June (Wang 2006). The



frequent rains may cause irreversible damage to pollen grains (Huang et al. 2002), including decreasing their viability and washing pollen grains from the anther, stigma or bodies of pollinators (Bynum and Smith 2001; Mao and Huang 2009; Wang et al. 2010). Indeed, the germination of pollen grains of *R. nobile* in distilled water was decreased by 88 % as compared to that in 10 % sucrose solution and most pollen grains, and sometimes entire anthers of flowers from which the bracts had been removed, were washed away by rain (Fig. S1f, g). As in *Davidia involucrata* (Sun et al. 2008), bracts concealing the whole inflorescence could act as “umbrellas” to reduce rain damage to the anthers and pollen grains.

#### Bracts enhance pollinator visits

Pollinators were found significantly more frequently on control plants than on plants with their bracts removed. Bracts are generally thought to improve pollination success by enhancing visual displays (Herrera 1997). In the field, the density of flowering plants of *R. nobile* was low, and only one or two plants may flower in some populations in some years, making it difficult for insects to find the flowering plants. The UV absorbance might make the bracts of *R. nobile* more conspicuous to pollinating insects (Kevan et al. 1996; Omori et al. 2000). This resembles the “detection” effect found for *D. involucrata* and *Salvia viridis*, in which the presence of bracts significantly increased pollinator visitation (Sun et al. 2008; Keasar et al. 2009). Keasar et al. (2009) observed that this “detection” effect was more prominent when the density of flowering plants was extremely low.

In general, the activity of pollinating insects is temperature dependent, and heat as a reward for pollinators has been described frequently (Kevan 1975; Cooley 1995; Herrera 1995; Totland 1996; Seymour and Schultze-Motel 1997; Dieringer et al. 1999; Sapir et al. 2006). For example, Nagy et al. (1972) and Knutson (1974) suggested that the bracts of *Philodendron* and *Symplocarpus* warmed the inflorescences to attract pollinating insects in the cold season. Therefore, the bracts of *R. nobile* may provide a warmer and more constant micro-environment for pollinators, protecting them from cold rain and strong winds.

#### Positive functions of bracts during seed development

Bad weather conditions (e.g., low temperature, high levels of UV, and heavy rain) have been found to affect seed development negatively (Nayyar et al. 2007; Kaur et al. 2008). Bract removal after flowering in *R. nobile* did, indeed, have a negative effect on seed development, adversely affecting fruit set, abortion rate, seed mass, and subsequent seed germination (Fig. 5a, b). The positive

effect of bracts during seed development may stem from a number of characteristics. First, seed development needs specific cumulative temperatures, to which cell division is sensitive (Egli and Wardlaw 1980; Boyer 1982; Kumar and Omae 2008). The bracts of *R. nobile* are able to increase the temperature of ripening fruits by as much as 8 °C above ambient levels on sunny days. In addition, Devlin and Witham (1983) suggested that higher temperatures could increase the rate of carbon transfer to the ovary from leaves and storage structures. Second, intense UV-B radiation can alter plant metabolism (Musil 1994, 1995; Harley et al. 1996) and result in biological damage during cell division and cell differentiation; this is particularly deleterious to nucleic acids (Caldwell 1981). Hence, development of the embryo might be adversely affected by the intense UV-B radiation after removal of bracts in *R. nobile*. Third, there is no doubt that bracts can protect fruits from physical damage by heavy rain, hailstones, and strong winds, which are frequent between June and September in the alpine region of the Himalayas.

#### Benefits and costs of bracts

Although the bracts of *R. nobile* greatly increased reproductive fitness, we also found that predation of seeds concealed by bracts was significantly higher than when the bracts were removed, i.e., bracts were associated with higher seed predation by larvae of *Bradysia* sp. feeding on the mature seeds. This is because *Bradysia* sp. is more likely to visit plants with bracts and to lay eggs on them. It is likely that the conditions provided by bracts facilitate the development of *Bradysia* sp. larvae. Another cost of bracts, which should be considered, is the investment in the bracts themselves: ca. 20 % of above-ground biomass during flowering. However, the percentage biomass of bracts decreases to ca. 6 % at the end of seed development due to the accumulation of biomass allocated to fruits (B. Song et al., unpublished data). Our results indicate that the overall reproductive success in the presence of bracts is much higher than reproductive success when bracts are removed either before or after flowering. Furthermore, like figs and fig wasps (Herre et al. 2008), a mutualism may exist between plant and insect: *Bradysia* sp. pollinates the plant and *R. nobile* provides brood sites and food for the insects. Thus, bracts may provide a nursery for its pollinator larvae, a fact that is usually ignored. Consequently, compared with the benefits, the costs associated with the bracts are low. In the harsh conditions at high elevations, the costly bracts of *R. nobile* provide substantial benefits for successful reproduction.

In conclusion, our comprehensive study reveals high functional benefits of bracts in *R. nobile*. In particular, we are the first to demonstrate experimentally the positive

function of bracts during seed development. Bracts of *R. nobile* enhance male fitness, i.e., by promoting pollen germination, protecting pollen grains from rain and intense UV-B radiation, and increasing visits by pollinators during flowering. They also enhance female fitness by facilitating the development of fertilized ovules during fruiting. The bracts are particularly important for seed development, since the contribution of bracts to reproductive success during fruiting is higher than during flowering: 28.2 vs. 15.3 %. In addition to the benefits, the bracts have an associated cost in terms of enhancing seed predation, but the costs are relatively modest. We conclude that the bracts of *R. nobile* represent an adaptive strategy, enhancing survival in the extreme alpine habitats of the Himalayas. Alpine plants in the Himalayas employ a variety of ways to survive particular environmental conditions, for example, nodding flowers, closing flowers by petal movements, flower heliotropism and changing floral orientation (He et al. 2006; Wang et al. 2010; Zhang et al. 2010b), indicating convergent evolution with respect to reproductive functions. However, to our knowledge, compared with other plants, the functions played by the bracts of *R. nobile* are the most diverse and provide a striking example of multiple and combined functions during and after flowering, as a result of selection pressure from both biotic and abiotic factors.

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