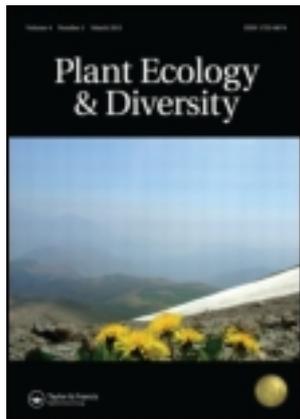


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Divergent seed production responses of white and blue flowers of *Gentiana leucomelaena* (Gentianaceae) to warming and watering

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Background: The adaptation, evolution and function of flower colour diversity in response to changing environments are one of the oldest puzzles in plant ecology. It is logical that comparative studies on fitness of flower colour would be the most meaningful if they were conducted on the same genotype.

Aims: We used *Gentiana leucomelaena*, which produces both white and blue flowers on different tillers of the same individual, as a model species to test adaption and fitness differences of contrasting flowers in contrasting environments.

Methods: We examined seed production by white and blue flowers in response to increased temperatures (28 °C/2 °C vs. 12 °C/2 °C; 12 h light/12 h dark) in a climate chamber study and to watering in the field (+1.5 l m⁻² d⁻¹ vs. control with no supplementary irrigation).

Results: For white and blue flowers warming decreased but watering increased seed number. Increased temperature and watering increased the size of seeds from white flowers, but neither warming nor watering significantly changed seed size of blue flowers. Seed size was significantly negatively correlated with seed number in the temperature treatments but positively correlated in watering treatments. The positive correlation was strong in white flowers, but the negative correlation was stronger in blue than in white flowers.

Conclusions: Water availability and low temperature confer an advantage to white flowers, while warming and dry habitats favour blue flowers. These divergent responses may influence total plant fitness and thus help explain the adaptive value and evolution of flower colour diversity.

Keywords: adaptation; flower diversity; reproductive strategy; seed number; seed size

Introduction

The evolution and function of flower diversity (e.g. colour, size, shape) is one of the oldest puzzles in plant ecology, and there has been much research on the different exogenous and endogenous determinants of flower diversity (Darwin 1859; Stebbins 1974; Galen 1999; Dormont et al. 2010). Previous studies on flower colour diversity often have focused on the effect of biotic factors on the evolution of the colour diversity, e.g. attracting insect pollinators (Ida and Kudo 2003; Chittka and Raine 2006; Kudo et al. 2007). Recently, with global climate change, an increasing number of studies have been concerned with the rapid adaptation and evolution of flower colour diversity to changing abiotic factors (Orenshamir and Levinissim 1997; Grace and Logan 2001; Steyn et al. 2002). However, there is a scarcity of experimental studies on the relative fitness of different flower colours under varying environmental conditions. The results of such studies would help us to better understand the evolution and function of flower colour diversity.

Previous studies on the function of flower colour diversity often have compared the fitness of individuals in different species or populations that had contrasting flower colours; however, due to genetic differences it is difficult to

determine the functional role of flower colour as a determinant of fitness. Theoretically, comparing the fitness between phenotypes within the same genotype (e.g. between tillers of the same individual) with contrasting coloured flowers is better than comparing genetically different members of a species with different colours. In plants with the same genotype, genetic variation would not be a constraining factor in determining the fitness of different flower colours. However, there are very few studies comparing the adaptation and fitness of contrasting flowers among individuals within a population or among tillers in the same individual. Flower polymorphisms are common in nature, e.g. both blue and white flowers are produced by *Linanthus parryae* (A. Gray) Greene, a California desert annual (Schemske and Bierzychudek 2007). Examples of species with tillers on the same plant that produce different colours of flowers are not common, but one example of such a species is *Gentiana leucomelaena* Maximowicz ex Kusnezow. In this species some tillers produce white flowers and others produce blue flowers (Figure 1). The use of *G. leucomelaena* as our experimental organism allowed us to control for the effect of genetic variation in comparative studies on the fitness (e.g. seed number and seed size) of flower colour and on responses of contrasting flower colours to climate

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Figure 1. The blue and white flowers of *Gentiana leucomelaena*.

change (e.g. warming and drying). To our knowledge, there have been no such attempts to explore the adaptation, evolution and functioning of flower colour diversity.

By using *Gentiana leucomelaena* as a model species, we made a series of experiments in a Tibetan alpine grassland to test for differences between blue and white flowers in response to various environmental conditions. In previous field studies, we found that white flowers were abundant in the cold and humid early spring with flowering lasting for two months, during which time flower pollination tended to be limited by lack of pollinators. In contrast, blue flowers dominated the relatively warm and dry late spring, during which time pollination seemed to be limited by pollinator competition (Mu et al. 2010, 2011). Based on these results, we predicted that tillers with white and blue flowers would differ in production of seeds (seed number and seed size) in response to simulated warming and watering. Additionally, we assumed that the relationship between seed number and seed size would differ in contrasting treatments and between white and blue flowers, due to different environmental and biological constraints (Baskin and Baskin 1998; Fenner and Thompson 2005; Moles et al. 2007). We tested these predictions by addressing two questions: (1) Do seed size and seed number of blue and white flowered tillers differ in response to warming and watering? (2) Do the relationships between seed number and seed size differ between white and blue flowers and among watering, warming and control treatments?

Materials and methods

Site and species

The experiment was conducted at the Hongyuan Alpine Meadow Ecosystem Research Station of Nanjing University (31° 50' to 33° 22' N and 101° 51' to 103° 23' E), located on the eastern edge of the Tibetan Plateau at 3494 m asl. Mean annual precipitation is 749 mm, and 80% of it occurs in the growing season from May to October. Mean annual temperature is 1.4 °C, and the mean maximum and minimum monthly temperatures in July and

January are 10.9 °C and –10.3 °C, respectively (Gao et al. 2008). Vegetation at the site is typical alpine meadow, with more than 95% cover and a mean canopy height of about 30 cm. Most species flower from early May to late August (detailed in Mu et al. 2010).

Gentiana leucomelaena is a fast growing annual herbaceous species belonging to *Gentiana* sect. *Chondrophyllae* (Figure 1). It grows along streams and in meadows and scrub in alpine regions of China, with its altitude ranging from 1900 m (at the eastern edge of the Tibetan Plateau) to 5000 m asl. The species is 5 to 10 cm in height with corollas averaging ca. 1.2 cm in diameter and 1.0 cm in length. The species is known for the production of both white and blue flowers on the same individual, and while the colour does not change with flower age, the relative abundance of the colours varies over the flowering season (Mu et al. 2011). Previous studies on the reproductive strategy of Tibetan alpine gentians have documented that whether flowers were pollinated by outcrossing or selfing often was dependent on flower abundance (He et al. 2006; Duan and Liu 2007; Duan et al. 2010). Our previous studies also found that this species was pollinated by selfing when pollinators were scarce in early flowering season, but outcrossing occurred when most flowers were open in the middle or late flowering stages and insects were relatively abundant (Mu et al. 2011). Furthermore, blue flowers often produced more pollen than white flowers, but blue flowers produced fewer ovules than did white flowers (Mu et al. 2011).

Warming and watering treatments

At the beginning of April 2011 when flowers started opening, we selected 360 individuals: 180 with white flowers (removed blue flowers) and 180 with blue flowers (removed white flowers) in the field and transplanted them into six containers (each with 60 4 cm × 4 cm × 8 cm cells). Each container included 60 individuals with 30 blue flowers and 30 white flowers, which were randomly assigned to 60 cells. Then, the six containers were allocated to two growth chambers (BIC800, Xinfu Electric Industrial Co. Ltd., Shanghai, China) set at a light intensity of 135 μmol m⁻² s⁻¹, 60% relative humidity, and a 12/12 h light/dark cycle. One chamber was set at a temperature regime of 28 °C/2 °C and the other at 12 °C/2 °C. The temperature values 28 °C, 12 °C and 2 °C were maximum day temperature, mean day temperature and mean night temperature in summer at the study site, respectively. Plants were regularly watered to keep the soil moist, which is a normal condition at the study site in May due to the thawing of snow and the falling of ca. 80 mm rain during this month (Mu et al. 2010). The high temperature and watering treatments (detailed below) were set up not only to simulate the current maximum temperature and soil moisture conditions in May at the study site, but also the projected future increased temperature and moisture conditions in summer on the eastern edge of the Tibetan Plateau (Lu and Liu 2010; Yang et al. 2012) where *Gentiana leucomelaena* is

still widely distributed. Therefore, the treatments approximate the gradients across which *G. leucomelaena* occurs in its total environmental range.

All flowers were covered by photic plastic bags before the stigmas matured. After flowering, pollen was taken from the blue or white flowers. Thirty blue flower stigmas were pollinated with pollen from blue flowers, and 30 white flowers were pollinated with pollen from white flowers. To test the effects of temperature on pollen maturation and on fertilisation, 30 blue flowers were pollinated with pollen from white flowers, and 30 white flowers were pollinated with pollen from blue flowers. Then, flowers were covered by photic plastic bags until the flowers withered. After three months of fruit ripening, seeds from each flower were counted and weighed with a 10^{-5} g precision balance. Seed size was calculated by dividing total mass of all seeds in a flower by the number of seeds produced by the flower. Biomass of each individual was weighed with a 10^{-4} g precision balance to indicate individual mass.

At the beginning of April 2011, 10 1 m \times 1 m plots with relatively abundant seedlings of *Gentiana leucomelaena* were established within a large (300 m \times 500 m) permanent plot in a *Kobresia*-dominated alpine meadow. These plots were used to monitor plant phenology in the field (detailed in Mu et al. 2010, 2011). Five plots were watered with 1.5 mm precipitation-equivalent water every day (at 16:00); the other five plots (control) were not watered and received only nature rainfall, ca. 80 mm precipitation during the experimental period (from mid-April to late May in 2011). As soon as plants flowered, we randomly selected six individuals for both blue and white flowers from each plot. Thus, 30 blue and 30 white flowered plants for watering/control treatment were selected, and the flowers were covered by photic plastic bags before the stigmas matured. When the stigmas matured, flowers were pollinated with pollen from flowers of the same colour. Then, flowers were covered by photic plastic bags until the flowers withered. The number and size of seeds per flower were determined as described above.

Data analysis

The data for seed number and size were log-transformed for fitting a normal distribution. We used seed number and size from each individual as a replicate by assuming that individuals independently produced seeds as (1) the individuals in each container were randomly assigned to the independent cells to grow; (2) the interaction between individuals sampled from field could be negligible due to the small size of *Gentiana leucomelaena* growth at relatively low density in the community; (3) each individual was covered by photic plastic bags during reproduction. Firstly, we used a three-level nested ANOVA to test the effect of treatment (low and high temperatures in growth chambers, and watered and control in the field), flower colour (blue and white flower) and plot (three containers in the growth chambers and five plots in the field) on seed number and seed size. We found that the effect of plot was not significantly

either in the warming or in the watering treatment. Then, we used 30 individuals with white/blue flowers in each treatment as replicates and performed a two-level nested ANOVA to test the effect of warming or watering (main factor) and flower colour (nested factor) on seed number and seed size. In both three- and two-level nested ANOVA, individual mass was used as an error term to adjust the effect of individual size on seed production. We used linear regression to test the correlation between seed size and seed number, and generalised linear model (GLM) to test the effect of flower colour and treatment (as fixed factor) on the relationship between seed number and seed size. All statistical analyses were carried out by using R 2.12.2 (R Development Core Team 2011).

Results

Overall, warming decreased the number of seeds produced per flower in both kinds of flowers, with white and blue flowers producing almost four-fold and double the number of seeds, respectively, at the lower temperature rather than at the higher temperature (Figure 2(a), Table 1). Watering significantly increased the number of seeds, but there was no significant difference between white and blue flowers in the increase in the number of seeds (Figure 2(b), Table 1).

White flowers produced larger seeds at the higher rather than at the lower temperature (Figure 3(a), Table 1) and in the watering treatment vs. the control (Figure 3(b), Table 1), while neither temperature nor watering significantly influenced seed size of blue flowers (Figures 3(a,b), Table 1).

For both white and blue flowers, seed size was significantly negatively correlated with seed numbers in the temperature treatments, but positively correlated in the watering treatments (Figure 4). The positive correlation was strong in white flowers (Figure 4(a)), and the negative correlation was stronger in blue than in white flowers (Figure 4(b)). Moreover, in the control treatment the correlation was very weak (for white flowers see Figure 4(a)) or not significant (for blue flowers see Figure 4(b)).

Discussion

Overall, in terms of seed production, water availability and low temperature confer advantages to plants of *Gentiana leucomelaena* with white flowers, while those with blue flowers were favoured more by warm and dry conditions. White flowers produced more and smaller seeds at the low temperature and more and larger seeds with watering. In contrast, seed size in plants with blue flowers was not significantly changed either by warming or by watering. These divergent responses of white and blue flowers to warming and watering may influence the total fitness of the species and thereby help explain the adaptive value of flower colour diversity.

Previous studies found that different floral pigments were often associated with tolerance to different environmental stresses, e.g. heat (Koes et al. 1990), drought

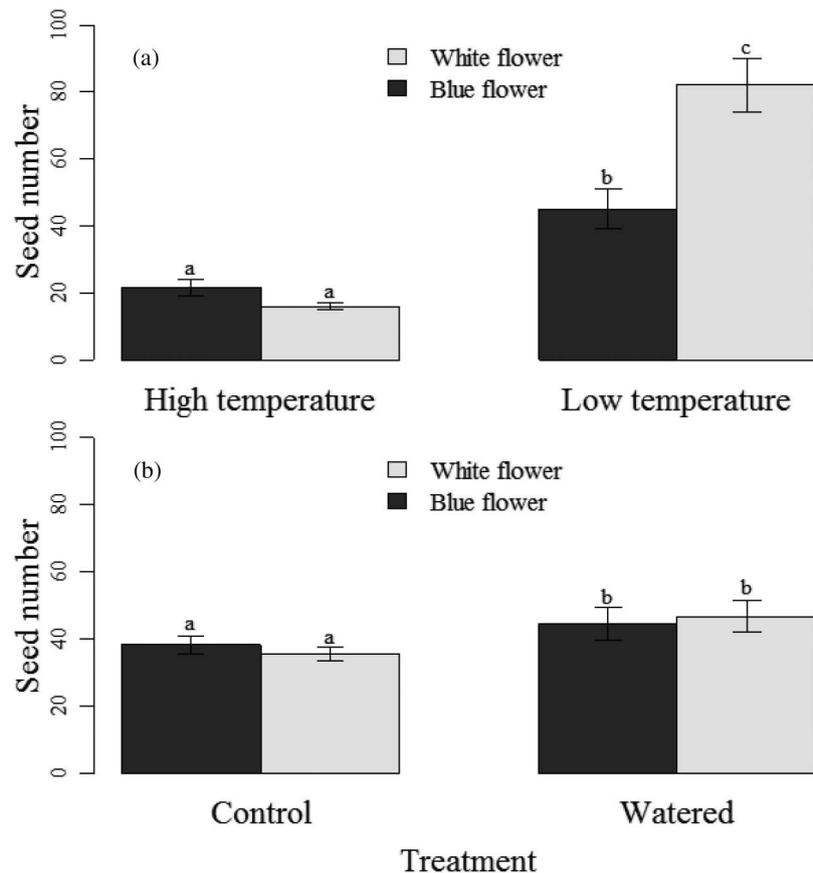


Figure 2. Changes in seed number in response to simulated warming (low and high temperature) and watering (control and watered). Values are given as mean \pm SE. Seed number differed significantly between the warming ($P < 0.01$) and watering ($P < 0.05$) treatments. Significant differences between flowers are indicated by different lowercase letters above bars assessed by two-level nested ANOVA (detailed in the data analysis and Table 1).

Table 1. Summary of two-level nested ANOVA for treatment effect (warming or watering) and flower colour on seed number and seed mass of *Gentiana leucomelaena*.

	Seed number				Seed mass			
	df	% SS	<i>F</i>	<i>P</i>	df	% SS	<i>F</i>	<i>P</i>
Warming								
Treatment (low/high temperature)	1	34.96	76.65	<0.001	1	28.72	63.26	<0.001
Treatment: flower colours (white/blue)	2	12.14	13.31	<0.001	2	18.61	20.49	<0.001
Residuals	116	52.90			116	52.67		
Watering								
Treatment (watered/control)	1	4.31	5.239	0.024	1	18.6	30.263	<0.001
Treatment: flower colours (white/blue)	2	0.34	0.209	0.811	2	10.0	8.12	<0.001
Residuals	116	95.35			116	71.4		

df, degree of freedom; % SS, percentage of sum of squares explained.

(Warren and Mackenzie 2001), spring rainfall (Schemske and Bierzychudek 2001), herbivores and pathogens (Frey 2004). Also, pigment production may be associated with pollen or ovule development and even fertilisation success (Coberly and Rausher 2003). Previous studies have shown that blue flowers often have multiple flavonoids in the petals, whereas white flowers may not produce flavonoids (Rausher 2008). Since flower pollination tends to be limited by pollinator competition in relatively late spring at our study site (Mu et al. 2011), flowers may allocate more

resources to producing pigment and larger flowers, such as blue flowers, which would attract pollinators (Figure 1, Figure S1). In contrast, relatively early spring flowers tend to be self-pollinated due to scarcity of pollinators (Mu et al. 2011), and plants produce anthocyanin-free and smaller flowers when temperatures are low (Figure 1, Figure S1, Table S1). These results are consistent with those from previous studies in which species with pigmented individuals often were more tolerant of water stress than anthocyanin-free morphs (Warren and Mackenzie 2001; Schemske and

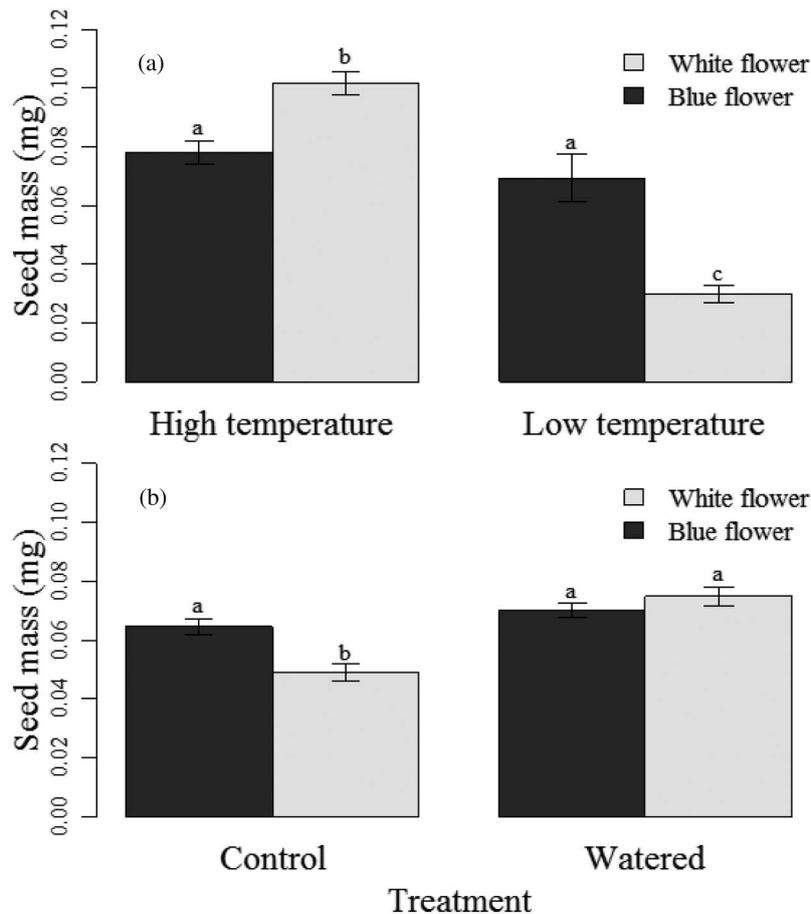


Figure 3. Changes in seed size in response to (a) simulated warming (low and high temperature) and (b) watering (control and watered). Values are given as mean \pm SE. Seed mass differed significantly between the warming ($P < 0.01$) and watering ($P < 0.01$) treatments. Significant differences between flowers are indicated by different lowercase letters above bars assessed by two-level nested ANOVA (detailed in the data analysis and Table 1).

Bierzychudek 2007). In short, these abiotic factors may indirectly influence the expression of flower colours, i.e. via pollinator limitation. Plants tend to save resources and produce seeds by self-pollination when pollinators are scarce in early spring, while they allocate more resources to producing pigment and larger flowers to attract pollinators in late spring (Mu et al. 2011).

With regard to the relationship between seed size and seed number, it is well-known that natural selection not only occurs at the species level but could also have an influence at the population and individual levels (Fenner and Thompson 2005). However, previous studies on flower colour often have focused on the species level. In our study, when we consider the population level there were no significant correlations between seed number and seed size in control (natural) or treatment (experimental), but significant correlations were observed under the experimentally manipulated temperature and watering treatments. These results suggest that, compared to natural conditions, experimental treatments changed resource constraints of seed production in both blue and white flowers of *G. leucomelaena*. Depending on the experimental temperature, flowers produced many small seeds or a few large ones, resulting in seed size being negatively correlated with seed

number at the population level. In contrast, watering may release plants from resource limitation on seed production, allowing large individuals with a greater capacity of fix carbon to produce many large seeds, while some small plants will produce a few small seeds (Westgate et al. 1989; Fenner and Thompson 2005). In this case, a positive correlation between seed size and seed number was observed (Figure 4). These results are consistent with those from many previous studies on the shift in relationship between seed number and seed size in response to environmental changes (reviewed in Reekie and Bazzaz 2005). It is meaningful to mention that the positive correlation was strong in white flowers (Figure 4(a)) but the negative correlation was stronger in blue than in white flowers. Thus, we conclude that plants with white flowers are better adapted to watering and low temperature conditions than those with blue flowers.

Additionally, at the higher temperature we failed to cross-fertilise white and blue flowers, and 70% and 95% of the white and blue flowers pollinated with pollen from blue and white flowers, respectively, did not produce seeds. These results suggest that high temperatures may influence the fitness of white flowers of *G. leucomelaena*. This is consistent with the prediction that anthocyanin-free flowers are

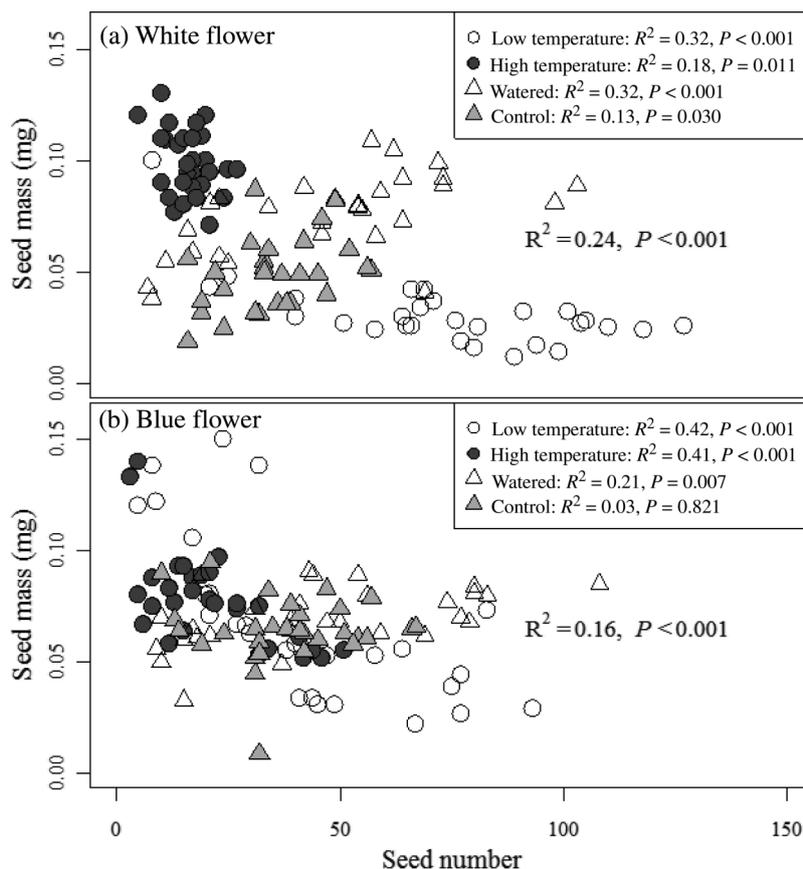


Figure 4. Relationship between seed size and seed number for (a) white flowers and (b) blue flowers overall (regression coefficient in subfigure) and at each treatment (regression coefficient in legend box). The markers indicate seed number or mean seed mass of each sampled individual. The R^2 and P values were estimated from linear regression.

better adapted to low-temperature stress in terms of development of pollen, ovules and fertilisation than those with high levels of pigments (Jewell et al. 1994; Seymour 2001; Ivancic et al. 2008; Li and Huang 2009; Mu et al. 2010).

Further work is required to examine the effects of fluctuating temperature or soil moisture on the evolution and functioning of flower diversity. In the present study, we only established two levels of treatment. The results from our experiments mainly reflect plant responses to specific treatments in a semi-natural habitat or in greenhouse conditions.

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Author contributions

KN and JM conceived and designed the experiments. JM and YP performed the experiments. KN and JM analysed the data. JM and YP contributed reagents, materials and analysis tools. KN and JM wrote the paper.

Notes on contributors

Junpeng Mu is a post-doctoral fellow. His research addresses the ecology and evolution of flower diversity in alpine regions.

Youhong Peng is an assistant professor. Her main research interest is evolution and function of alpine plants in response to climatic change.

Kechang Niu is an assistant professor. His research interests focus on the pattern and process of biodiversity assembly in alpine grassland.

Supplementary material

Supplementary material is available for this article.

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