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Original Article

Linkages among the functional traits, insect visitation rate and seed set of *Gentiana* taxa on the Tibetan Plateau

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Abstract: Maximum plant height (H_{max}), stem tissue mass density (SD), leaf mass per area (LMA), and relative growth rate (RGR) have all been linked to flowering phenology. However, it is still unknown whether these functional traits varying with flowering phenology are related to other floral traits associated with pollinator preference and reproductive success. We investigated the relationship between vegetative and floral traits, as well as the rates of insect visitation and seed set of five *Gentiana* species in eastern Tibetan meadows. Our results showed that flowering onset and flowering offset time were all found to be positively correlated with the H_{max}, SD, and LMA, but negatively correlated with the RGR. Flowering onset time was positively correlated with corolla diameter and pollen grain number, whereas was negatively correlated with flower number and flower longevity. The rates of insect visitation were positively related to flowering onset time, pollen grain number, corolla diameter, and seed set, but negatively related to flower number and longevity. Early flowering species have higher RGR but lower SD, LMA and H_{max}, as well as smaller flowers, fewer pollen grains, longer flower longevity, and lower insect visitation rates than late-flowering plant species. Our findings indicate that floral traits are related to vegetative traits in *Gentiana* species. Also these plant trait relationships were associated with pollinator preference, and plant reproductive success of eastern Tibetan meadows. We speculate that these traits relationships are likely adaptive in unpredictable and often pollinator-limited environments in the Tibet alpine meadows.

Keywords: Life history strategy; Mating system; Reproductive efficiency; Alpine plant

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1 Introduction

Outside of the developmental and phylogenetic constraints that may limit the evolution of floral traits (Donoghue et al. 1998; Sargent et al. 2007), these traits may respond to biotic and abiotic factors (König et al. 2018; Nock et al. 2016; Wang et al. 2020). Several authors suggest that plants produce nourishing substances (e.g., pollen and nectar, see Mu et al. 2014a; Vaudo et al. 2016, 2020) and evolve a variety of flower traits (e.g., flowering phenology and single-flower longevity, flower shape and colour, flower size and display size, flower scent, see Sahli and Conner 2011; Kulbaba and Worley 2012; Barberis et al. 2021) as adaptations to pollinators (Vaudo et al. 2016, 2020; Ågren 2019; Parachnowitsch et al. 2019). Some studies suggest that floral traits could be determined by abiotic factors (e.g., temperature, soil nutrients and water, elevational gradient; see Rathcke and Lacey 1985; Pélabon et al. 2013; Junker and Larue-Kontić 2018; Dai et al. 2022).

Some vegetative traits are related to flowering phenology (Du and Qi 2010; Liu et al. 2021). For instance, species that reach maximum plant height (H_{max}) early are more likely to emerge earlier, grow faster, and flower earlier (e.g. high relative growth rate, RGR) than species that reach maximum plant height later (Du and Qi 2010; Sun and Frelich 2011). Leaf mass per area (LMA) is often associated with the nutrient concentration and photosynthetic capacity of leaves, and it has been widely recognized to have a negative relationship with RGR, suggesting that plants with low LMA can increase leaf area and maximize light interception, as well as maintain a high photosynthetic capacity and relative growth rate (Hunt and Cornelissen 1997). Stem tissue mass density (SD) represents plant dry mass per unit fresh stem, reflecting a trade-off among growth rate, plant height and shoot quality (Liu et al. 2021). Previous studies have shown that flowering onset time is positively correlated with H_{max}, LMA, and SD but negatively correlated with RGR (Sun and Frelich 2011; Liu et al. 2021). This suggests that vegetative traits are associated with flowering phenology. However, it is still unclear whether vegetative traits (e.g. H_{max}, LMA, RGR and SD) vary with flowering phenology are related to other floral traits, such as flower size, flower number, and flower rewards (e.g., quality and quantity of nectar and pollen).

Flowering traits influence pollinator activity and availability, which affect plant reproduction. Flower colour, shape, scent, and number, as well as nectar rewards and guides, all influence the pollinator visitation rates of resource extraction and plant reproduction (Geber et al. 2006). For example, a positive relationship between nectar volumes and pollinator visitation, and seed set has been found in many species (Real and Rathcke 1991; Mitchell 2004; Mu et al. 2011). In alpine regions, early-flowering individuals of *Gentiana leucomelaena* often have longer flower longevity, lower pollinator visitation rates, and a higher ability to self-pollinate autonomously than later-flowering individuals (Mu et al. 2011). This suggests that flower traits were related to pollinator preferences, which influence plant reproductive success. We deduced that H_{max}, LMA, RGR, and SD vary with flowering phenology were related to flower size and number, and pollen grain number, which associated with pollinator preference and seed production (e.g., seed production and seed set).

Gentiana is the most widely distributed genus on the Tibetan Plateau, and it has a longer flowering phenology. Previous studies have primarily focused on local adaptations at the species level (He et al. 2005; Mu et al. 2011; Dai et al. 2022), with little attention given to the relationships among vegetative and floral traits, pollinator visitation rate and seed production. Herein, we investigated the relationships among the vegetative (e.g., H_{max}, LMA, RGR and SD) and floral traits (e.g., flowering onset and offset time, nectar volume and pollen grain number, flower size, flower number and longevity), insect visitation rate and seed traits (seed production and seed set) of five sympatric *Gentiana* species (*Gentiana squarrosa, G. leucomelaena, G. spathulifolia, G. aristata and G. formosa*) with the aim of describing the trait relationships and possible consequences to the reproductive success of *Gentiana* species on the eastern Tibetan Plateau.

2 Study Site and Study Species

This study was conducted in an alpine meadow in Hongyuan County on the eastern Tibetan Plateau (32°48′N and 102°33′E). The altitude is 3200-3600 m. The climate is cold, with a long frosty winter and a short and cool spring, summer, and autumn. The mean annual precipitation is 749 mm, with 80%

falling between May and October. The annual mean temperature is 0.9°C, with maximum and minimum monthly means of 10.9°C and -10.3°C in July and January, respectively. Sedges, grasses, and forbs dominate the meadows. The total vegetation coverage is greater than 95%, and the plant height is ~30 cm. The soil has a high organic matter content and a low availability of nitrogen (N) and phosphorus (P). The growing period lasts from May to September. Detailed information about the climate, soil conditions and vegetation are presented by Mu et al. (2011) and Hu et al. (2019).

Gentiana squarrosa, *G. leucomelaena*, *G. spathulifolia*, *G. aristata* and *G. formosa* herbs (Fig. 1) grow in alpine regions of China at elevations ranging from 1900 (at the eastern edge of the Tibetan Plateau) to 5000 m asl. Their height ranged from 5 to 17 cm, and they produced 4 to 25 shoots, each with one flower. Flowering phenology varies among species: *Gentiana squarrosa*, *G. leucomelaena,* and *G. spathulifolia* are early flowering species (from early March to late May), whereas *G. aristata* and *G. formosa* were late-flowering species (blooming in July-August and September, respectively). Fruits mature from late April to mid-October. Flies, bumblebees, and honeybees are their main pollinators (Mu et al. 2011, 2014a; Hu et al. 2019; Dai et al. 2022)

3 Materials and Methods

3.1 Measurements of vegetative traits

The tallest plant was used to calculate the maximum plant height, which was labelled H_{max}. *Gentiana* species produce 4-25 shoots, and each shoot has one flower located at the top of the shoot. As a result, we measured the flower height relative to the ground surface as the maximum plant height during the peak flowering of each species (Heady 1957). We measured the height of each shoot and took the tallest shoot as the maximum plant height. The shoot height was determined using a scale ruler (0.1 cm). In 2015, we chose 25 plots at random to monitor vegetative and floral traits. Three plants of each species were randomly selected in each plot (2 m×2 m) to monitor the plant height from March to September.

The leaf mass per area (LMA) was calculated by dividing the leaf dry mass by the fresh leaf area (Sun and Frelich 2011). From March to September, we collected mature and fully developed leaves from three individual plants in each plot. All leaves of each plant were selected and scanned before being oven-dried for 48 hours at 65°C. All of the leaves in this study came from areas of plants that received direct sunlight. The sample size was the same as it was in H_{max}.

The stem tissue mass density (SD) was calculated for each individual by cutting 1-cm-long segments from stems at 1 cm above the ground surface. The long (a) and short (b) axes of each segment were measured with the assumption that the stem cross section was elliptical in shape. The fresh volume was calculated by multiplying $\pi ab/4$ by the segment length (Sun and Frelich 2011). Here, $\pi ab/4$ was the cross-sectional area, where a and b were the long and short axes, respectively. The segment length was 1 cm. Each segment was dried for 48 h at 65°C and weighted (0.01 mg; MeilenMCS220, China); SD was expressed as dry mass per fresh volume (mg·mm^{3}; Sun and Frelich 2011).

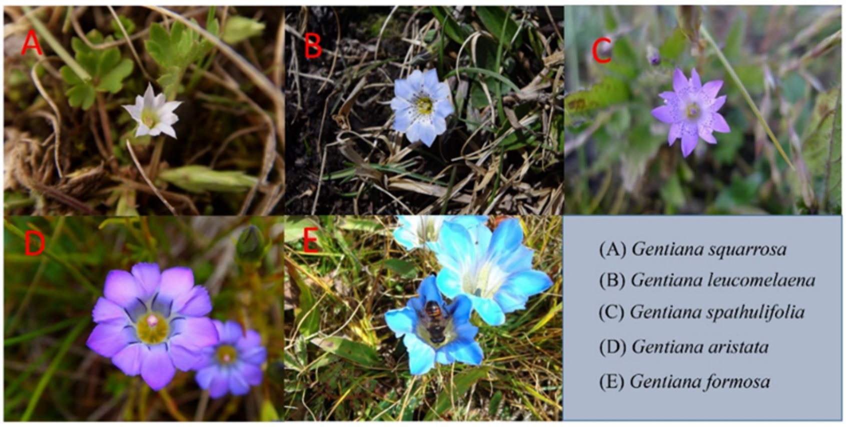

Fig. 1 Flowers of *Gentiana* species in eastern Tibetan Plateau.

The relative growth rate (RGR) was calculated as = $(\ln(W_2) - \ln(W_1))/(t_2-t_1)$, W_1 and W_2 were the plant dry weights at times t_1 (the leaf expansion period for each species) and t_2 (the squaring stage for each species), following the procedure of Hunt (1982). We collected three plants per species in each plot (2 m × 2 m) after seedling establishment, dried them to constant mass at 65°C, weighed them to dry mass as W_1 (0.1 mg), and recorded the date (t_1). We sampled and recorded the date (t_2) during the squaring stage.

Leaves were removed from plants and stored in a portable icebox with dry ice before being transported to the laboratory to estimate nonstructural carbohydrate levels according to Hansen and Møller (1975) and Yoshida et al. (1976). For all previous measurements, 25 plots (2 m×2 m) were selected, with three plants from each species planted in each plot (n=375 plants).

3.2 Measurements of flower traits

Flowering phenology was defined as the times when the first individual plant began flowering and the last individual plant finished flowering, which is the flowering onset and offset time, respectively (Hovenden et al. 2008). From March to September, we recorded the first and last individual plant flowering of each species in each plot. The flower longevity was monitored from March to September. Ten flowers of each species were labelled in each plot, and the dates when they first opened and when they wilted were recorded. Flower longevity was calculated as the time span between two dates (Mu et al. 2011; n=625 flowers).

To estimate pollen grain production, three flowers of each species were collected after flower opening when anthers were immature in each plot from March to September. The anthers were removed and dried on paper at 20°C for 24 – 48 hours. Pollen grains were then immersed in an aniline-blue lactophenol solution. Using a dissecting microscope, we counted the number of pollen grains in each flower (Cruden 1977).

To record the number of flowers in each plant, five plants of each species were labelled in each plot from March to September. We counted the number of opened flowers on each plant every day and labelled them with red twine strings. The total number of flowers per plant was the sum of the number of flowers per day (from the first flower opened to the last flower closed for the individual plant).

Corolla diameter was defined as the distance between the tips of the opposite corolla lobes of fully opened flowers (Galen 1989). Ten flowers from each species were measured with digital callipers (ABSOLUTE Digimatic Calliper Series 500; Mitutoyo) with a precision of 0.01 mm.

3.3 Insect visitation rate

From March to September, the insect visitation rate was tracked during each species' peak blooming period. Two plants from each plot were chosen at random. For flies and bees, the observation distance was approximately 3 m, and for ants, it was approximately 1 m (Mu et al. 2011). The distinction between legitimate and illegitimate floral visitors was determined by whether the visitors contacted the stigmas (Li et al. 2015). Observation periods were evenly distributed between 9:00 and 17:00 per day. During each hour, one person observed each individual plant for one minute from a fixed point (50 individual plants ×1 min). Two individual plants in each plot was observed per hour due to the presence of two observers. The observations were only conducted out on sunny days, and each observation was replicated five sunny days per plot. A total of 4000 minutes of field observations were accumulated for each species. The observation experiment was conducted using the methods of Vaudo et al. (2014) and Mu et al. (2011). Visitation rates (R) were calculated as the total number of legitimate visits (Nv) divided by the number of flowers (Nc) and the observation time (minutes), following the protocols of Arroyo et al. (1985, $R = Nv/(Nc \times min)$).

3.4 Measurements of seed traits

To examine the seed set of each species under field conditions, we randomly selected 10 flowers from each plot during the peak blooming period of each species from March to September. White plastic labels were used to label each flower. As each fruit matured, seeds were collected, counted individually for each fruit, and weighed to 0.01 mg using a digital balance (MeilenMCS220, China). All seeds per fruit were weighted to calculate seed production. The seed set was calculated as viable *seeds*/ (*viable seeds + aborted seed number*) (Mu et al. 2011). Seed mass was calculated by dividing seed production per flower by the number of flower seeds.

3.5 Statistical analysis

Before proceeding with the analysis, the data for each trait were averaged for each plot. Relationships among the traits studied were determined with correlation analysis and regression curves and the above-mentioned parameters were fitted using the method of least squares (Sun and Frelich 2011). The corrplot package was used to identify correlations among vegetative traits, floral traits, insect visitation rates, and seed traits. All statistical analyses were performed using R (R Development Core Team 2020).

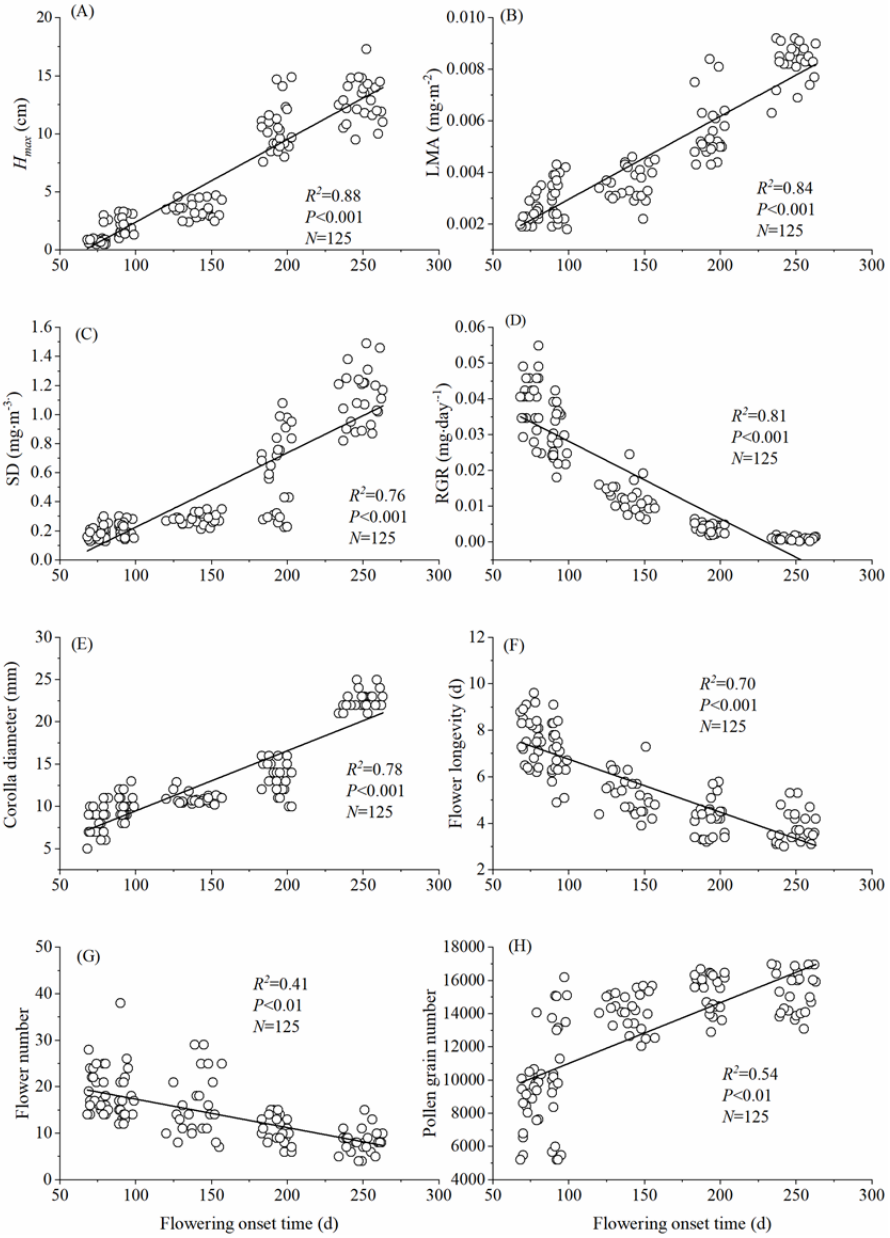

Fig. 2 Relationships of flowering onset time versus (A) maximum plant height, (B) leaf mass per area, (C) stem tissue mass density, (D) relative growth rate, (E) corolla diameter, (F) flower longevity, (G) flower number, and (H) pollen grain number in *Gentiana* species.

4 Results

Flowering onset and flowering offset time were all positively correlated with H_{max}, SD, LMA, and nonstructural carbohydrates, but negatively related to RGR (Table 1, Figs. 2 - 3).

Flowering onset and flowering offset time were all positively correlated with corolla diameter and pollen grain number, but negatively correlated with flower number and flower longevity (Table 1, Fig. 2). There was a positive relationship between nonstructural carbohydrates and pollen grain number (Table 1).

Early flowering species had low insect visitation rate, whereas late flowering species had high insect visitation rate. There was a positive relationship between flowering onset time and insect visitation rate (Fig. 4).

Insect visitation rate was positively correlated with corolla diameter and pollen grain number, but negatively correlated with flower longevity and flower number (Fig. 5). There was a positive relationship between seed set and insect visitation rate (Fig. 4). Seed production and seed number were positively correlated with flowering onset and offset time (Table 1).

5 Discussion

Our findings support the hypothesis that vegetative traits are associated with flower traits in *Gentiana* taxa, and that these relationships are related to visitation rate and plant reproduction in Tibetan meadows.

Table 1 Correlation matrix showing the correlation coefficients among vegetative and flower traits, insect visitation rates, seed traits of *Gentiana* species on the Tibetan Plateau

	H_{max}	RGR	LMA	SD	NSC	FONT	FOFFT	FN	FL	PN	CD	VR	SS
H_{max}													
RGR	-0.84^{***}												
LMA	0.93^{***}	-0.78^{***}											
SD	0.90^{***}	-0.71^{***}	0.91^{***}										
NSC	0.85^{***}	-0.87^{***}	0.83^{***}	0.75^{***}									
FONT	0.94^{***}	-0.90^{***}	0.92^{***}	0.87^{***}	0.90^{***}								
FOFFT	0.94^{***}	-0.87^{***}	0.93^{***}	0.88^{***}	0.89^{***}	0.99^{***}							
FN	-0.66^{***}	0.61^{***}	-0.63^{***}	-0.60^{***}	-0.63^{***}	-0.65^{***}	-0.65^{***}						
FL	-0.78^{***}	0.82^{***}	-0.71^{***}	-0.67^{***}	-0.81^{***}	-0.84^{***}	-0.82^{***}	0.57^{***}					
PN	0.67^{***}	-0.77^{***}	0.59^{***}	0.54^{***}	0.69^{***}	0.74^{***}	0.71^{***}	-0.53^{***}	-0.77^{***}				
CD	0.84^{***}	-0.72^{***}	0.90^{***}	0.87^{***}	0.80^{***}	0.89^{***}	0.90^{***}	-0.58^{***}	-0.69^{***}	0.58^{***}			
VR	0.93^{***}	-0.84^{***}	0.90^{***}	0.86^{***}	0.91^{***}	0.95^{***}	0.95^{***}	-0.66^{***}	-0.78^{***}	0.68^{***}	0.88^{***}		
SS	0.63^{***}	-0.60^{***}	0.63^{***}	0.59^{***}	0.63^{***}	0.65^{***}	0.67^{***}	-0.47^{***}	-0.54^{***}	0.49^{***}	0.60^{***}	0.65^{***}	
SP	0.19^{ns}	-0.26^{*}	0.36^{**}	0.31^{*}	0.28^{*}	0.31^{*}	0.34^{*}	0.11^{ns}	-0.19^{ns}	0.19^{ns}	0.43^{***}	0.27^{*}	0.49^{***}

$^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$, ^{ns} non-significant correlations. H_{max}, maximum plant height; RGR, relative growth rate; LMA, leaf mass per area; SD, stem tissue mass density; NSC, non-structure carbohydrates; FONT, flowering onset time; FOFFN, flowering offset time; FN, flower number; FL, flower longevity; PN, pollen grain number; CD, corolla diameter; VR, visitation rate; SS, seed set; SP, seed production.

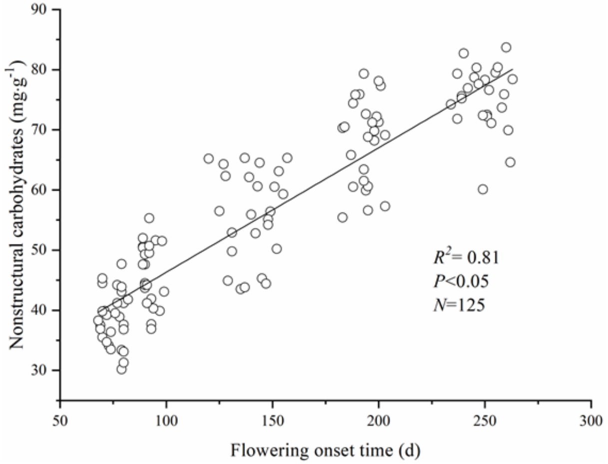

Fig. 3 Relationship of flowering onset time versus nonstructural carbohydrates in *Gentiana* species.

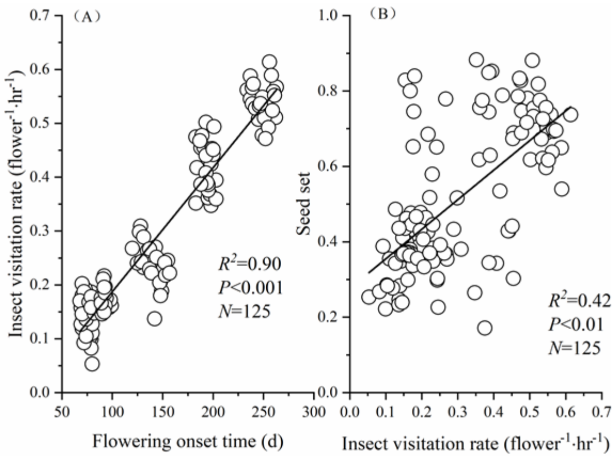

Fig. 4 Relationship of flowering onset time versus pollinator visitation rate (A), and pollinator visitation rate versus seed set (B) in *Gentiana* species.

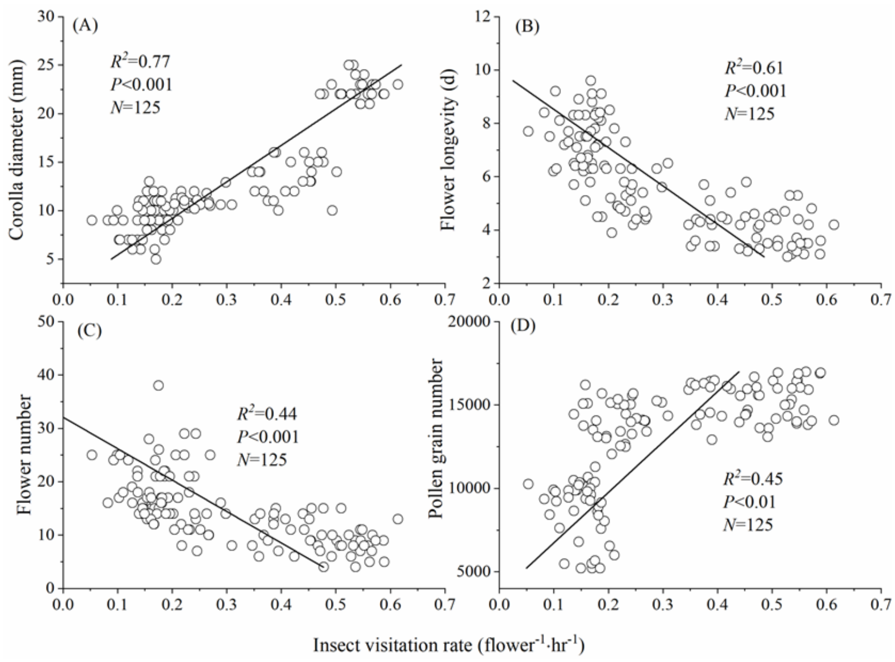

Fig. 5 Relationships of insect visitation rate versus (A) corolla diameter, (B) flower longevity, (C) flower number and (D) pollen grains number in *Gentiana* species.

5.1 Relationship between vegetative traits and flower traits

Our findings show that vegetative traits have a significant impact on flowering phenology. A high LMA is widely accepted to be associated with a low photosynthetic capacity of leaves (Hunt and Cornelissen 1997). Early flowering *Gentiana* species have a higher RGR but a lower LMA than later flowering *Gentiana* species. This implies that early flowering species have a high photosynthetic capacity in their leaves, resulting in a high relative growth rate. *Gentiana* species generally require a larger amount of light, and competition for light is intensive (e.g. *G. leucomelaena*. see Mu et al. 2011). Early flowering species (*G. squarrosa* and *G. leucomelaena*) require open habitats to produce sufficient photosynthates for growth. Later flowering species (*G. aristata* and *G. formosa*) were much shorter (plant height was 3.5 ± 0.46 cm, but community height was 15.7 ± 0.39 cm, Unpublished data) between leafing and flowering, suggesting that close habitats diminish photosynthates for growth, and delay flowering phenology. Later flowering species have a higher H_{max} than early flowering species (Bolmgren and Cowan 2008). Taller plants and flowers attract more pollinators to visit them (Althoff et al. 2005). Flowering onset time is positively correlated with stem tissue mass density, and SD is positively correlated with H_{max}, implying that later flowering species with high SD are taller than early flowering species with low SD.

Carbohydrate levels (starch and sugars) are related to the amount of pollen grains (Sheoran and Saini 1996). Temperature-induced carbohydrate loss, for example, reduces the amount of pollen grains (Shaked et al. 2004; Firon et al. 2006). In this study, there was a positive relationship between pollen grain number and nonstructural carbohydrates (Table 1). Early flowering *Gentiana* species have a low nonstructural carbohydrates, resulting in a small number of pollen grains (Table 1, Fig. 3). On the other hand, later flowering *Gentiana* species have a high nonstructural carbohydrates, which produced more pollen grains (Table 1, Fig. 3). Generally, pollinators are less common in alpine meadows during the early flowering season than during the later flowering season (Kudo 1993; Mu et al. 2011). Early flowering species evolved autogamous flowers with few pollen grains, whereas later flowering species evolved cross-pollination flowers with more pollen grains. This suggests that variations in pollen grain number of *Gentiana* species may be related to adaptations to lower pollinator availability in alpine meadows.

Flower longevity was inversely related to flowering onset time. Flower longevity is frequently influenced by pollinator visitors. Early flowering species have a longer time of pollen grain maturation, delaying flower longevity (Mu et al. 2011). However, later flowering species have a shorter pollen grain maturation time and more pollinators, which reduce flower longevity, suggesting that flower onset time associated with flower longevity.

Corolla diameter is positively correlated with flowering onset time. Early flowering species invest more photosynthates into reproductive growth, particularly in the flower mass fraction (early flowering species was (57.3 ± 0.77)%, whereas late flowering species was (25.6 ± 1.21)%. F=16.04, P<0.01, Unpublished data). Furthermore, the tradeoffs are demonstrated by the negative relationships between flower number per plant and corolla diameter (Table 1). Early flowering species invest more resources to increase the number of flowers, but not the size of the flowers. Later flowering plant species have large flowers that require a high SD for support.

Later flowering species put more effort into increasing vegetative growth, particularly plant height and stem tissue mass density. However, early flowering species invest more resources to increase reproduction, particularly flower number (Fig. 2). These tradeoffs are demonstrated by the negative relationships between flower number per plant and H_{max}, and flower number per plant and stem tissue mass density (Table 1).

5.2 Relationship between flower traits and the insect visitation rates

Pollinator visitation rates are thought to be related to flower rewards (e.g., the quality and quantity of nectar and pollen; see Fenster et al. 2006; Vaudo et al. 2016). Pollen is the primary source of proteins and lipids for bees and flies, so higher pollen quality and quantity of pollen attracts more pollinators (Norris 1965; Young and Stanton 1990; Vaudo et al. 2014 and 2016; Muth et al. 2016). In our study, visitation rates of legitimate floral visitors, including flies, bumble bees, and honey bees (Mu et al. 2011, 2014a, 2014b; Dai et al. 2022) were found to be positively correlated with pollen grain number in our study.

In this study, flowering onset time was found to be positively correlated with insect visitation rates. In early flowering species, infrequent and unpredictable insects resulted in a low insect visitation rate. On the other hand, later flowering species received more frequent visitors pollinators prefer large and numerous flowers (Fenster et al. 2006). Insect visitation rates were found to be positively correlated with corolla diameter. A previous study demonstrated that larger flowers have a higher nectar volume (Fenster et al. 2006). In this study, although nectar was produced in undetectable amounts in early flowering species, we discovered that later flowering species, *G. formosa,* produced larger and nectar-rich flowers and show the greatest visitation rate of all *Gentiana* species. This suggests that larger flowers with more nectar attracted more insects.

5.3 Relationship between insect visitation rate and seed set

The frequency of pollinator visits determines seed set in many flowers (Waites and Ågren 2004; Mu et al. 2011; Dai et al. 2022; Su et al. 2022). The insect visitation rate was found to be positively correlated with seed set. In this study, early flowering species with smaller flowers, fewer pollen grains, and longer flower longevity associated with self-pollination appear to be adapted to infrequent and unpredictable pollinators, whereas later flowering species seems to produce larger flowers, many pollen grains, and short flower longevity to adapt to the greater abundance of pollinators (Du and Qi 2010).

5.4 Implications for traits linkage of Gentiana species

The flowering for *Gentiana* species ranged from March to September. Floral traits that vary with flowering phenology are linked to vegetative traits that adapt to unpredictable pollination. To adapt to a low abundance of pollinators, early flowering species produce smaller flowers, fewer pollen grains, and longer flower longevity, with high self-pollination. Later flowering species may entice more pollinators to visit flowers (Kühsel and Blüthgen 2015). This suggests that the trait linkage of *Gentiana* species may be related to pollinator preference and seed production in Tibetan meadows. In addition, *Gentiana* species display a mixed mating system (a mixture of selfing and outcrossing; see Petanidou 2001; Mu et al. 2011). The degree to which individuals are outcrossing and selfing reflects reproductive assurance, inbreeding depression, and genetic diversity (Lloyd 1965; Levin 2012). When pollen transfer is limited, the plant produces many self-fertile seeds (Broz et al. 2017). We found early-flowering species had more self-fertilized seeds (16.3 ± 1.01 seeds per flower) than later-flowering species (3.5±0.89 seeds per flower. F=14.52, P<0.01, Unpublished data). This is consistent with the low pollination-insect density during the early flowering season and the high pollination-insect density during the late flowering season. Despite the fact that there are many co-flowering species in the late flowering season, late flowering species receive more visitors than early flowering plants. This implies that early flowering species face greater pollen limitation than late-flowering species. In this study, we still do not know the inbreeding depression and genetic diversity of *Gentiana* species, which may influence species adaptation. More research will need to be conducted to determine the effects of inbreeding depression and genetic diversity in *Gentiana* species on plant adaptation. The life forms of the species sampled here were diverse (e.g., annual herbs vs. perennial herbs). We still do not know whether these life forms have any effect on the relationships between vegetative traits and flower traits. Future investigations will examine the relationships between vegetative traits and floral traits with different life forms. These plant trait relationships were found to be associated with pollinator preference and plant reproductive success in eastern Tibetan meadows. Furthermore, plant developmental and evolutionary restrictions may alter these traits. Future studies is needed to see if these findings can be generalized to other genus species and habitats.

6 Conclusion

Our findings suggest that floral traits are related to vegetative traits in *Gentiana* species. Floral traits that change with flowering phenology are linked to vegetative traits within-habitat species of *Gentiana* taxa that adapt to unpredictable pollination in eastern Tibetan meadows. Despite the discovery of trait correlations in *Gentiana* species, the cause and effect of between functional traits and flower traits (e.g., flower longevity and H_{max}) remain unknown.

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References

Ågren J (2019) Pollinators, herbivores, and the evolution of floral traits. Science 364(6436): 122-123. https://doi.org/10.1126/science.aax1656

Althoff DM, Segraves KA, Pellmyr O (2005) Community context of an obligate mutualism: Pollinator and florivore effects on Yucca filamentosa. Ecology 86(4): 905-913. https://doi.org/10.1890/04-1454

Arroyo MTK, Armesto JJ, Primack RB (1985) Community studies in pollination ecology in the high temperate Andes of central Chile. II. Effect of temperature on visitation rates and pollination possibilities. Plant Syst Evol 149(3): 187-203. https://doi.org/10.1007/BF00983305

Barberis M, Bogo G, Bortolotti L, et al. (2021) Gender-biased nectar targets different behavioral traits of flower visitors. Plant Ecol 222: 233-246. https://doi.org/10.1007/s11258-020-01101-5

Bolmgren K, Cowan PD (2008) Time-size tradeoffs: a phylogenetic comparative study of flowering time, plant height and seed mass in a north-temperate flora. Oikos 117(3): 424-429. https://doi.org/10.1111/j.2007.0030-1299.16142.x

Brown VK, Lawton JH (1991) Herbivory and the evolution of leaf size and shape. Philos T R Soc B 333(1267): 265-272. https://doi.org/10.1098/rstb.1991.0076

Broz AK, Randle AM, Sianta SA, et al. (2017) Mating system transitions in Solanum habrochaites impact interactions between populations and species. New Phytol 213(1): 440-454. https://doi: 10.1111/nph.14130

Cruden RW (1977) Pollen-ovule ratios: a conservative indicator of breeding systems in plants. Evolution 31:32-46. https://doi.org/10.2307/2407542

Dai WF, Yang YL, Patch HM, et al. (2022). Soil moisture affects plant-pollinator interactions in an annual flowering plant. Philos T R Soc B 377(1853): 20210423. https://doi.org/ 10.1098/rstb.2021.0423

Donoghue M, Ree RH, Baum DA (1998) Phylogeny and the evolution of flower symmetry in the Asteridae. Trends Plant Sci 3(8):311-317. https://doi.org/10.1016/S1360-1385(98)01278-3

Du GZ, Qi W (2010) Trade-offs between flowering time, plant height, and seed size within and across 11 communities of a QingHai-Tibetan flora. Plant Ecol 209(2): 321-333. https://doi.org/10.1007/s11258-010-9763-4

Fenster CB, Cheely G, Dudash MR, et al. (2006) Nectar reward and advertisement in hummingbird-pollinated *Silene virginica* (Caryophyllaceae). Am J Bot 93(12): 1800-1807. https://doi.org/10.3732/ajb.93.12.1800

Firon N, Shaked R, Peet M, et al. (2006). Pollen grains of heat tolerant tomato cultivars retain higher carbohydrate concentration under heat stress conditions. Sci Hortic 109, 212-217. https://doi.org/10.1016/j.scienta.2006.03.007

Galen C (1989) Measuring pollinator-mediated selection on morphometric floral traits: bumblebees and the alpine sky pilot, *Polemonium viscosum*. Evolution 43(4): 882-890. https://doi.org/10.1111/j.1558-5646.1989.tb05185.x

Geber MA, Moeller DA, Harder LD, et al. (2006) Pollinator responses to plant communities and implications for reproductive character evolution, Ecology and Evolution of Flowers, Oxford University Press. pp 102-119.

Hansen J, Møller IB (1975) Percolation of starch and soluble carbohydrates from plant tissue for quantitative determination with anthrone. Anal Biochem 68(1): 87-94. https://doi.org/10.1016/0003-2697 (75)90682-x

He YP, Duan YW, Liu JQ, et al. (2005) Floral closure in response to temperature and pollination in *Gentiana straminea* Maxim. (Gentianaceae), an alpine perennial in the Qinghai-Tibetan Plateau. Plant Syst Evol 256(1/4): 17-33. https://doi.org/10.1007/s00606-005-0345-1

Heady FH (1957) The measurement and value of plant height in the study of herbaceous vegetation. Ecology 38(2): 313-320. https://doi.org/10.2307/1931691

Hovenden MJ, Wills KE, Schoor JKV, et al. (2008) Flowering phenology in a species-rich temperate grassland is sensitive to warming but not elevated CO_2. New Phytol 178(4): 815-822. https://doi.org/10.1111/j.1469-8137.2008.02419.x

Hu L, Wu XW (2019) The difference in pollen harvest between

Apis mellifera and *Apis cerana* in a Tibetan alpine meadow. J Mt Sci-Engl 16: 1598-1605. https://doi.org/10.1007/s11629-018-5245-2

Hunt R (1982) Plant growth curves. The functional approach to plant growth analysis. London: Edward Arnold.

Hunt R, Cornelissen JHC (1997) Components of relative growth rate and their interrelations in 59 temperate plant species. New Phytol 135(3): 395-417. https://doi.org/10.1046/j.1469-8137.1997.00671.x

Junker RR, Larue-Kontić AAC (2018) Elevation predicts the functional composition of alpine plant communities based on vegetative traits, but not based on floral traits. Alpine Bot 128:13-22. https://doi.org/10.1007/s00035-017-0198-6

König P, Tautenhahn S, Cornelissen JHC, et al. (2018) Advances in flowering phenology across the Northern Hemisphere are explained by functional traits. Global Ecol Biogeogr 27(3): 310-321. https://doi.org/10.1111/geb.12696

Kudo G (1993) Relationship between flowering time and fruit set of the entomophilous alpine shrub, *Rhododendron aureum* (Ericaceae), inhabiting snow patches. Am J Bot 80(11): 1300-1304. https://doi.org/10.1002/j.1537-2197.1993.tb15368.x

Kühsel S, Blüthgen N (2015) High diversity stabilizes the thermal resilience of pollinator communities in intensively managed grasslands. Nat Commun 6(1): 7989. https://doi.org/10.1038/ncomms8989

Kulbaba MW, Worley NC (2012) Selection on floral design in *Polemonium brandegeei* (Polemoniaceae): female and male fitness under hawkmoth pollination. Evolution 66(5): 1344-1359. https://doi.org/10.1111/j.1558-5646.2011.01536.x

Levin DA (2012) Mating system shifts on the trailing edge. Ann Bot-London 109(3): 613-620.http://doi.org/10.1093/aob/mcr159

Li JK, Song YP, Xu H, et al. (2015) High ratio of illegitimate visitation by small bees severely weakens the potential function of heteranthery. J Plant Ecol 8(2): 213-223. https://doi.org/10.1093/jpe/rtv021

Liu YZ, Li GY, Wu XW, et al. (2021) Linkage between species traits and plant phenology in an alpine meadow. Oecologia 195(2):409-419. https://doi.org/10.1007/s00442-020-04846-y

Lloyd DG (1965) Evolution of self-compatibility and racial differentiation in Leavenworthia (Cruciferae). Contributions of the Gray Herbarium Harvard University 195: 3-134. https://doi.org/10.1093/aob/mcr159

Mitchell RJ (2004). Heritability of nectar traits: why do we know so little. Ecology 85(6): 1527-1533. https://doi.org/10.1890/03-0388

Mu JP, Li GY, Niklas KJ, et al. (2011) Difference in floral traits, pollination, and reproductive success between white and blue flowers of *Gentiana leucomelaena* (Gentianaceae) in an Alpine Meadow. Arct Antarct Alp Res 43(3): 410-416. https://doi.org/10.1657/1938-4246-43.3.410

Mu JP, Peng YH, Xi XQ, et al. (2014a) Domesticated honeybees evolutionarily reduce flower nectar volume in a Tibetan Asteraceae. Ecology 95(11): 3161-3172. https://doi.org/10.1890/13-2055.1

Muth F, Francis JS, Leonard AS (2016) Bees use the taste of pollen to determine which flowers to visit. Biol Letters 12(7): 20160356. https://doi.org/10.1098/rsbl.2016.0356

Nicolson SW, Wright GA (2017) Plant-pollinator interactions and threats to pollination: perspectives from the flower to the landscape. Funct Ecol 31(1): 22-25. https://doi.org/10.1111/1365-2435.12810

Nock CA, Vogt RJ, Beisner BE (2016) Functional Traits. In: eLS. John Wiley & Sons, Ltd: Chichester. https://doi.org/10.1002/9780470015902.a0026282

Norris K (1965) The bionomics of blow flies. Annu Rev Entomol 10(1): 47-68. https://doi.org/10.1146/annurev.en.10.010165.000403

Parachnowitsch AL, Manson J S, Sletvold N (2019) Evolutionary ecology of nectar. Ann Bot-London 123(2): 247-261. https://doi.org/10.1093/aob/mcy132

Pélabon C, Osler N, Diekmann M, et al. (2013) Decoupled phenotypic variation between floral and vegetative traits: distinguishing between developmental and environmental correlations. Ann Bot-London 111(5): 935-944. https://doi/10.1093/aob/mct050

Petanidou T (2001) Differential pollination success in the course of individual flower development and flowering time in *Gentiana pneumonanthe* L. (Gentianaceae). Bot J Linn Soc 135(1): 25-33. https://doi/10.1006/bojl.2000.0356

R Core Team (2020) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.R-project.org/

Rathcke B, Lacey EP (1985) Phenological patterns of terrestrial plants. Annu Rev Ecol Syst 16(1): 179-214. http://www.jstor.org/journals/annrevs.html

Real LA, Rathcke BJ (1991). Individual Variation in Nectar Production and Its Effect on Fitness in Kalmia Latifolia. Ecology 72(1): 149-155. https://doi.org/10.2307/1938910

Sahli HF, Conner JK (2011) Testing for conflicting and non-additive selection: floral adaptation to multiple pollinators through male and female fitness. Evolution 65(5): 1457-1473. https://doi.org/10.1111/j.1558-5646.2011.01229.x

Sargent RD, Goodwillie C, Kalisz S, et al. (2007) Phylogenetic evidence for a flower size and number trade-off. Am J Bot 94(12): 2059-2062. https://doi.org/10.3732/ajb.94.12.2059

Shaked R, Rosenfeld K, Pressman E (2004) The effect of low night temperatures on carbohydrates metabolism in developing pollen grains of pepper in relation to their number and functioning. Sci Hortic-Amsterdam 102(1): 29-36. https://doi.org/10.1016/j.scienta.2003.12.007

Sheoran IS, Saini HS (1996) Drought-induced male sterility in rice: Changes in carbohydrate levels and enzyme activities associated with the inhibition of starch accumulation in pollen. Sexual Plant Reprod 9(3): 161-167. https://doi.org/10.1007/BF02221396

Su RJ, Dai WF, Yang YL, et al. (2022) Introduced honey bees increase host plant abundance but decrease native bumble bee species richness and abundance. Ecosphere 13(6): e4085. https://doi.org/10.1002/ecs2.4085

Sun SC, Frelich LE (2011) Flowering phenology and height growth pattern are associated with maximum plant height, relative growth rate and stem tissue mass density in herbaceous grassland species. J Ecol 99(4): 991-1000. https://doi.org/10.1111/j.1365-2745.2011.01830.x

Vaudo AD, Patch HM, Mortensen DA, et al. (2014) Bumble bees exhibit daily behavioral patterns in pollen foraging. Arthropod-Plant Inte 8(4): 273-283. https://doi.org/10.1007/s11829-014-9312-5

Vaudo AD, Patch HM, Mortensen DA, et al. (2016) Macronutrient ratios in pollen shape bumble bee (Bombus impatiens) foraging strategies and floral preferences. P Natl Acad Sci US A 113(28): E4035-E4042. https://doi.org/10.1073/pnas.1606101113

Vaudo AD, Tooker JF, Patch HM, et al. (2020) Pollen Protein: Lipid Macronutrient Ratios May Guide Broad Patterns of Bee Species Floral Preferences. Insects 11(2): 132. https://doi.org/10.3390/insects11020132

Waites AR, Ågren J (2004) Pollinator visitation, stigmatic pollen loads and among population variation in seed set in *Lythrum salicaria*. J Ecol 92(3): 512-526. https://doi.org/10.1111/j.0022-0477.2004.00893.x

Wang ZH, Chlus A, Geygan R, et al. (2020) Foliar functional traits from imaging spectroscopy across biomes in eastern North America. New Phyto 228(2): 494-511. https://doi.org/10.1111/nph.16711

Young HJ, Stanton ML (1990) Influences of floral visitation on pollen removal and seed production in wild Radish. Ecology 71(2): 536-547. https:// doi.org/10.2307/1940307

Yoshida S, Forno D, Cock J, et al. (1976) Laboratory manual for physiological studies of rice. Manila, Philippines: International Rice Research Institute.