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The optimization of seed yield across the flowering season of *Gentiana leucomelaena* (Gentianaceae), an herbaceous Tibetan annual

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Abstract

The seed yield of herbaceous annuals may be resource- or pollinator-limited. The extent to which either or both of these limitations negatively affect seed yield is hypothesized to vary with the timing of flowering because of seasonal changes in pollinator and resource availabilities. We tested this hypothesis by examining the relationship between seed yield and pollinator visitation rates, flowering phenology, aboveground plant biomass, and biomass allocation patterns, fruit set, and seed set, seed number per flower, and seed mass for *Gentiana leucomelaena*, an annual herbaceous dicot that produces four types of flowering shoots as a result of differences in flower color (blue and white) and dichogamy-type (protogyny and protandry). Bivariate plots of reproductive efficiency (total seed mass per shoot) versus vegetative mass were curvilinear and showed that early- and late-flowering shoots were more likely to be limited in seed yield than shoots flowering during the middle of the flowering season. Early-flowering shoots had greater biomass but had lower fruit set and seed set with fewer but larger seeds per fruit. These features were consistent with pollinator (visitation rate) limitations on seed yield. In contrast, late-flowering shoots had smaller biomass but had higher fruit set and seed set with many but smaller seeds per fruit. These features were consistent with resource (plant biomass) limitations on seed yield. Moreover, early-flowering shoots tended to produce white and protogynous flowers and allocated more to leaf growth, whereas late-flowering shoots tended to produce blue and protandrous flowers and allocated more to stem growth in height (perhaps to reduce shading by neighbors). In addition, the peak time of flowering shoot density coincided with the peak of reproductive efficiency. In conclusion, limitations on the seed yield of this species varied with the timing of flowering. *G. leucomelaena* appears to have evolved a system of changing flower color and dichogamy-type that optimizes seed yield in an alpine meadow habitat.

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Introduction

Many spring ephemerals grow under high light intensities and low temperatures during early spring (Lapointe, 2001) and complete their vegetative growth and reproduction in a short time (e.g., ≈ 2 months in temperate deciduous forests) before canopy closure (e.g., Lapointe, 2001). For these plants, the optimization of seed yield is important for population regeneration and species persistence, particularly for ephemerals living in cold alpine habitats. Theoretically, there are at least two major limiting factors for seed yield for these kinds of plants. One is a limitation on aboveground plant biomass (i.e., “resource limitation”; see Zimmerman and Aide, 1989) resulting from low temperatures during a short growing season (Campbell and Halama, 1993; Lapointe, 2001; Du and Qi, 2010). Indeed, among herbaceous annuals living in cold alpine habitats, mature plant height typically does not exceed 10 cm (McKenna and Houle, 2000a) and seed numbers are low (Klinkhamer et al., 1987; see, however, Wilson and Thompson, 1989). The second limiting factor for seed yield, particularly for insect-pollinated alpine forbs, is a low abundance of pollinators and associated insufficient pollen transfer during the early spring (i.e., “pollinator limitation” or “pollen risk”; Bierzychudek, 1981; Kudo, 1993; Molau, 1993; Kudo et al., 2004). Accordingly, some alpine species have evolved specific strategies

such as autogamy to cope with low or unpredictable pollinator availability or activity (Kudo, 1993).

An additional confounding factor is that the relative importance of resource and pollinator abundance may vary with the timing of flowering, particularly if the flowering season is sufficiently long and if the habitat is unpredictable or mercurial. For example, compared to their early-flowering conspecifics, late-flowering plants may not be pollinator-limited provided that the pollinator abundance progressively increases during the growing season (Kudo, 1993; Totland, 1994, 1997; Kudo et al., 2004; but see Nishikawa and Kudo, 1995). Correspondingly, late-flowering plants might gain an advantage over early-flowering plants by evolving allogamy, as for example by changing the proportion of protogyny and protandry in flower populations (Lloyd and Webb, 1986; Bernhardt and Thien, 1987), especially among alpine and Arctic species (Bertin and Newman, 1993; Webb and Pearson, 1993). The counterpoint to this strategy is that late-flowering plants often experience shade stress due to more vigorously growing neighboring plants (provided that reproductive allocation reduces vegetative height growth). Under these circumstances, slow growth rates and resource accumulation rates are predicted to limit seed yield (Hughes, 1992).

Previous studies have explored the effect of physical factors (including temperature, light, and water availability) on seed yield and

have demonstrated the importance of resource limitation and pollinator limitation (Campbell and Halama, 1993; McKenna and Houle, 2000a, 2000b; Badri et al., 2007; Brookes et al., 2008). However, the relative importance of each limitation during different phases of a moderately long flowering season remains unclear. To redress this, we focused on *Gentiana leucomelaena*, a widespread, short annual herbaceous forb species on the Tibetan Plateau, whose flowering season ranges from late March to late May or early June. This species has two flower colors (blue and white) that occur within a population as well as within individual plants. In addition, each of the two flower colors may be either protandrous or protogynous, resulting in four different flower types or “morphs.” Previous studies have shown that the white flowers of this species blossom earlier than the blue flowers and that the blue flowers are more likely to be pollinated by insects such as honeybees (*Apis cerana*), flies, ants, and moths (Mu et al., 2010, 2011), which might be an adaptation to the rarity of pollinators in early spring in alpine areas (Kudo, 1993; Kudo et al., 2004). In order to further explore the reproductive ecology of this species, we measured average plant aboveground biomass, the frequency of flowering shoots, the proportion of protogyny and protandry, insect visitation rates, biomass allocation, and seed yield for both flower-color morphs under natural and hand-pollinated conditions at three flowering stages. Our primary objective was to determine whether limitations on seed yield vary with the timing of flowering. As noted above, large plants can potentially produce more and larger seeds than small ones, but this may not happen when pollinators are rare (in the absence of selfing). Likewise, it is very unlikely that small plants will produce more and larger seeds compared to larger plants even if pollinators are abundant and pollen limitation is absent. We therefore surmised that seed yield would be pollination-limited when seed production was observed for large plants and when pollinators were scarce, particularly if hand pollination improved seed production (see also Zimmerman and Aide, 1989). Likewise, if hand pollination did not improve seed production and plant biomass was small (see also Janzen et al., 1980; Montalvo and Ackerman, 1987), seed yield was assumed to be resource-limited. We hypothesized that early-flowering shoots, especially those with blue flowers, would be more likely to be pollinator-limited, whereas late-flowering shoots would be more likely resource-limited (as reflected by small plant biomass) because these shoots are often shade-stressed by taller earlier growing shoots. In addition, because protogynous flowers are more likely to produce seeds (due to high selfing rates) when pollinators are scarce compared to protandrous flowers, we also hypothesized that early-flowering shoots will tend to be protogynous.

Methods

STUDY SITE

This study was conducted in an alpine meadow of Hongyuan County, Sichuan Province of eastern Qinghai-Tibetan Plateau, China (32°48'N, 102°33'E). The altitude is around 3500 m. The climate is characterized by a short spring and autumn, a long winter, and a mild but cool summer. The mean annual precipitation is 690mm, 80% of which occurs in the growing season between May and October for most of the plant species in the study site. The mean annual temperature is 0.9 °C, with maximum and minimum monthly means of 10.9 °C and −10.3 °C in July and January, respectively (Liu et al., 2011). The vegetation is dominated by *Agrostis trinii*, *A. hugoniana*, *Potentilla anserina*, and *Anemone trullifolia* var. *linearis*. Total vegetation coverage is more than 90%, with an average plant height of ~30 cm. The soil is characterized by a high organic content (250 g·kg^{−1}) and low total N (8 g·kg^{−1}) and P (5 mg·kg^{−1}).

SPECIES DESCRIPTION

Gentiana leucomelaena is an annual herbaceous species in the *Gentiana* sect. *Chondrophyllae*. As a spring ephemeral, seedlings emerge and grow in late fall and the following winter. Plants are typically 5–10 cm tall and bloom between March and May, when few other species flower in the study site. Fruits mature from late April to middle June. Each plant typically produces five to fifteen shoots, each producing one flower; not all the flowers bloom at the same time—some shoots/individuals may flower after the aboveground parts of others die off. The species produces two colors of flowers (blue and white) and three types of plants in the study population, that is, individuals with only blue or white flowers, and individuals with flowers of both colors. Blue-flower plants have a trait combination (Mu et al., 2011) that typically attracts insects (primarily honeybees) and thus achieve a high rate of cross-fertilization. In contrast, white-flower plants characteristically have a high selfing rate (Mu et al., 2011). Moreover, this species is often dichogamous and each flower may be protandrous or protogynous. Thus, this species has four types of floral “morphs” (blue vs. white flowers × protandrous vs. protogynous flowers).

MEASURING FLOWERING PHENOLOGY

We established five 2 × 2 m plots in a meadow in March 2011. From 31 March (when the first flowers were observed) to 25 May 2011, all the flowers within each plot were numbered and tagged once they emerged. We visited each plot and tagged blossoming flowers using differently colored strings. We then recorded flower numbers for both colors every five days. We also recorded whether each flower was protogynous or protandrous following the protocols of Lloyd and Webb (1986); the dichogamy type was visually determined while the flower was open. Flower type was recorded once only for each flower. There were a total of eleven censuses over a period of 56 days. For simplicity, we artificially divided the flowering period into three stages based on the percentage of opened flowers in the plot—that is, an early stage (from 31 March to 10 April) during which about 10% of all flowers opened, a middle and peak stage (from 11 April to 14 May) during which 80% of all flowers opened, and a late stage (from 15 May to 25 May) during which about 10% of all flowers were opened.

MEASURING PLANT BIOMASS AND BIOMASS ALLOCATION

Aboveground biomass was used to indicate resource availability. Plant biomass and biomass allocation were measured at three times (31 March, 15 April, and 15 May) during which we randomly harvested aboveground biomass (excluding previously dead basal leaves) of 30 healthy shoots for each of the four flower morphs after measuring shoot height (height of photosynthetic tissue relative to ground surface; see Heady, 1957). Each shoot was divided into stems, leaves, and flowers in the lab. The samples were dried at 75 °C for 48 hr and then weighed to the nearest 0.001mg. Shoot biomass was the sum of stem, leaf, and flower mass.

INSECT VISITATION RATES

During the flowering season, five 2 × 2 m plots were established on 28 March 2001, and five persons simultaneously surveyed the plots for potential pollinators and visitation frequencies on five sunny days during each of the three flowering stages. During each survey, we recorded pollinator species (bees, flies, ants, and the

lepidopterans such as butterflies) and the number of flowers each visited hourly for each of the four flower morphs and for each plot (from 9:00 to 17:00). The observation distance was about 3 m for fly, bee, and lepidopteran species and about 1 m for ant species. Visitation rates were calculated as the total number of visits divided by the observation time and divided by the number of flowers observed following the protocols of Arroyo et al. (1985) and Mu et al. (2011).

MEASURING SEED YIELD

At the end of each of the three flowering stages, a minimum of twenty ripe fruits were harvested from sites outside the plots for each of the four flower types or “morphs.” A fruit was considered to be mature if its pod emerged from its closed corolla (pods enclosed by corollas emerge as soon as their seeds mature; see Mu et al., 2011). Seeds were collected and individually counted for each fruit (from June to July) and weighed to 0.001 mg. Empty seeds were considered to be aborted. “Seed yield” was measured as seed mass per flower. Seed mass (mg) was calculated as the total seed mass per flower divided by the seed number per flower.

HAND POLLINATION EXPERIMENTS

To examine the extent to which pollination limitation affects seed yields in the three different flowering stages, we tagged 20 shoots for each of the four flower types and emasculated flowers soon after they first opened but before anther dehiscence and stigma receptivity (as indicated by the opening of bilobed stigmas bearing feather-like trichomes). Flowers were pollinated under natural conditions in five plots; in the other five plots, flowers were bagged and pollinated by hand using pollen from other individuals as soon as stigmas were receptive. Hand-pollinated flowers were subsequently bagged until the flowers closed permanently. After

fruits ripened, we recorded fruit number and seed number, and dried and weighed the seeds.

DATA ANALYSIS

Fruit set and seed set were used to quantify the efficiency of pollinator visitation. Fruit set was calculated as the percentage of the number of fruits divided by the number of flowers per shoot; seed set was calculated as the percentage of ovules maturing into viable seeds. The fractions of flower, leaf, and stem biomass allocation (shoot biomass allocation) were also calculated with respect to total aboveground biomass to characterize shoot biomass allocation patterns. Additionally, total seed mass per shoot divided by the sum of vegetative and flower biomass was used to characterize reproductive efficiency for each of the three flowering stages.

Data for each of the measured traits were tested for normality before analysis. The data for the fruit set and seed set, and for the fractions of biomass allocated to leaves, stems, and flowers were \log_{10} -transformed to achieve normality. We used three-way ANOVAs to determine the effects of flower color, dichogamy-type (protogyny vs. protandry), and stage (early, middle, and late) on flower morphology, biomass allocation patterns, plant height, fruit set and seed set, total seed mass, and seed number and size at the shoot level. All statistical analyses were performed in R (R Development Core Team, 2011; <http://www.R-project.org/>).

Results

FLOWER PHENOLOGY

The relationship between flower number per plot and time was curvilinear for each of the four flower types or “morphs” (Fig. 1). The peak flowering times also differed among the four morphs. The flowering time of white and protogynous flowers peaked

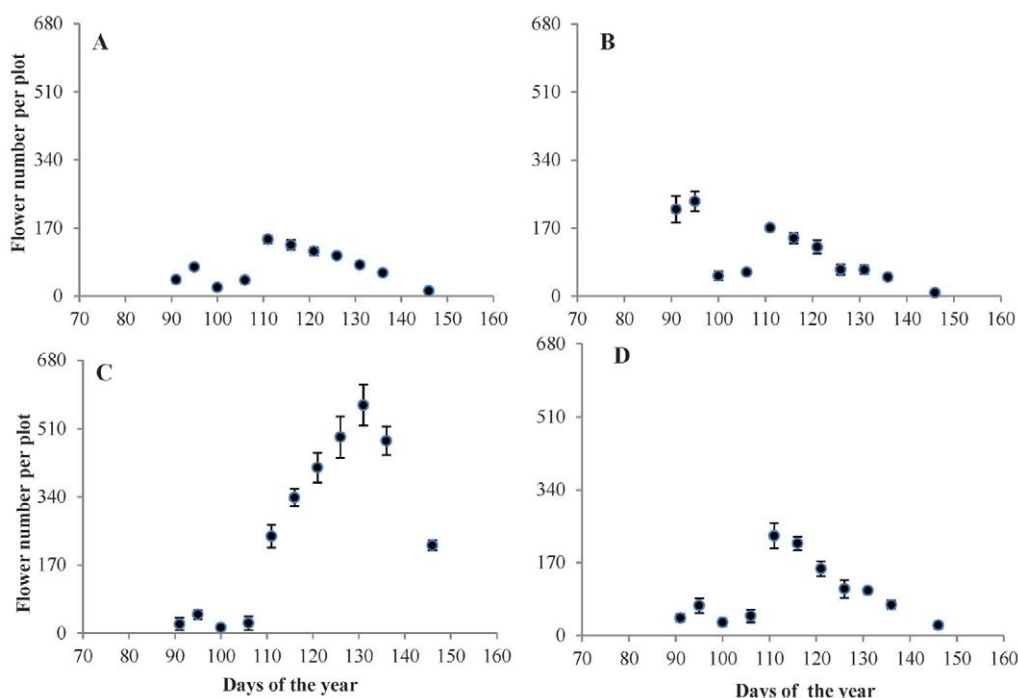


FIGURE 1. Means (± 1 SE) of (A) the number of blue and protogynous flowers, (B) white and protogynous flowers, (C) blue and protandrous flowers, and (D) white and protandrous flowers during the flowering season. $n = 5$.

during the end of March 2011, which was about 20 days earlier than either the blue and protogynous or the white and protandrous flowers, which peaked in the middle of April (Fig. 1, parts A and D). Blue and protandrous flowers peaked in early May (Fig. 1, part C). For the total population, about 80% of all shoots flowered from the middle of April to the middle of May.

PLANT BIOMASS AND BIOMASS ALLOCATION

Shoot biomass and plant height markedly differed during the flowering season (Tables 1 and 2). The biomass of early-flowering shoots was almost twice that of the late-flowering shoots, while plant height of late-flowering shoots was more than five times that of early-flowering shoots. Shoots producing white flowers had, on average, a smaller stem biomass fraction than shoots with blue flowers. Shoots with protandrous flowers had a larger aboveground biomass than those with protogynous flower morphs, although the difference between the flowers differing in color was indistinguishable statistically.

The leaf biomass fraction decreased over the flowering period in contrast to the stem biomass fraction, which increased over time. Plants flowering in the late flowering stage allocated over seven times more biomass to stems than plants flowering during the early stage, although the biomass fraction of flowers remained comparatively constant over the entire flowering season.

INSECT VISITATION RATES

Visitation rates changed markedly during the flowering season (Fig. 2). Pollinators were generally absent during the early flowering stage; ants and flies did not appear until the middle flowering stage (e.g., ants were not observed visiting flowers until 12 April 2011). During the middle flowering stage, honeybees were the primary visitors, followed by flies and ants particularly for protandrous flowers (Fig. 2, part A); lepidopterans also occasionally visited flowers. During the late flowering stage, however, the visitation rate of flies was the highest among all potential pollinators (Fig. 2, part B), and the visitation rates of flies, ants, and honeybees were all lower compared to those at the middle flowering stage (Fig. 2, part A).

The preferences of pollinator species for the four different flower types differed over the three flowering stages. Honeybees preferred blue and protandrous flowers during the middle and late flowering stages, whereas flies and ants favored blue and white protandrous flowers (Fig. 2). Protogynous flowers had comparatively low visitation rates regardless of color.

SEED YIELD

Except for the white and protogynous floral morph, the remaining three morphs had the highest seed mass per fruit during the middle flowering stage (Fig. 3, part A). The white and protogynous flowers had the highest seed mass per fruit during the early flowering time, the blue and protandrous floral morph had the highest seed mass per fruit in the middle stage, and the blue and protogynous had the highest seed mass per fruit in the late stage. A similar trend was also found in seed number per fruit (Fig. 3, part B). Individual seed mass decreased from the early and middle stages to the late stage in all the floral morphs with the exception of the white and protandrous morph (Fig. 3, part C): the differences among the morphs were statistically nonsignificant between the early and middle stages for all the four flower types. These

observations collectively indicated that early flowering shoots tended to have few but large seeds per fruit and that late-flowering shoots were more likely to have few and small seeds per fruit (relative to the shoots produced during middle stage of flowering).

Fruit set was significantly enhanced by hand pollination in the early and late stages of flowering for all flower types (with the exception of the white and protogynous floral type). Under natural conditions, fruit set was generally the lowest in the early stage and highest in the middle stage for all flower types with the exception of the white and protogynous morph, which manifested the highest in the early stage (Fig. 4, part A). With hand pollination, however, all flower types had higher fruit set in the early and middle stages compared to the late stage (Fig. 4, part B). A similar trend was also observed for seed set, which was higher during the early and middle flowering stages compared to the late stage (Fig. 4, parts C and D).

Finally, reproductive efficiency was the highest during the middle flowering stage compared to all other stages, with the exception of the white and protogynous flower type, which peaked during the early flowering stage (Table 2).

Discussion

The window of opportunity for growth and reproduction is often very limited for spring-flowering ephemeral annuals. For these species, growth and reproduction present a challenge to maximize reproductive success because pollinators are generally scarce and temperatures are sufficiently low to limit carbon accumulation (McKenna and Houle, 2000b; Kudo et al., 2004). Our study illustrates this challenge by showing that the effects of pollinator and resource limitation change over the growing season of *Gentiana leucomelaena* and alter the reproductive efficiency of this species differentially in response to the type of flowers produced on shoots. Thus, overall, our data provide support for the resource and pollinator limitation hypothesis.

Specifically, the reproductive success of *G. leucomelaena* changes curvilinearly during the flowering season such that early and late flowering plants appear to be limited in their seed productivity compared to plants flowering during the middle of the flowering season (Fig. 5). Early-flowering shoots are characteristically large but have a low fruit set with few but large seeds per fruit under natural pollination conditions. Hand pollination significantly enhanced fruit and seed set for all four types of flowers during the early-flowering season, which we interpret to reflect a pollinator and pollen limitation on seed yield. In contrast, late flowering shoots tend to be small and have more but smaller seeds per flower as well as high fruit set and seed set (relative to the early stage of flowering). Moreover, hand pollination did not significantly elevate fruit and seed set for any of the four types of flowers, which we interpret to reflect a resource-limiting syndrome. Consistent with the intensities of these two major limitations of contrasting trends over the course of the flowering season, we observe that seed yield peaks during the middle flowering stage (Fig. 5). We also observe that early-flowering shoots are more likely to bear white and protogynous flowers, whereas late-flowering shoots tend to produce blue and protandrous flowers. These observations are consistent with a spectrum of functional traits that improve seed yield. For example, white and protogynous flowers are more successful during the early spring of the flowering season because these morphs are highly selfing.

TABLE 1

Results of three-way ANOVAs showing the effects of dichogamy-type (protogyny vs. protandry), flower color (white vs. blue), and flowering stage (early, middle, and late) on flower number per plot, plant height, aboveground plant biomass, flower mass fraction, stem mass fraction, leaf mass fraction, total seed mass per flower, seed mass, total seed number per flower, and seed set ratio for *G. leucomelaena*.

	Source	df	F
Flower number per plot	Color (C)	1	513.07***
	Type (T)	1	1047.55***
	Stage (S)	10	241.83***
	C × T	1	1253.57***
	S × T	10	197.39***
	S × C	10	177.15***
	C × S × T	10	71.84***
	Error	176	
Shoot height	Color (C)	1	0.525***
	Type (T)	1	24.411***
	Stage (S)	2	554.754
	C × T	1	0.253
	S × T	2	0.702
	S × C	2	1.441
	C × S × T	2	0.061
	Error	348	
Aboveground plant biomass	Color (C)	1	0.177***
	Type (T)	1	33.034***
	Stage (S)	2	120.569
	C × T	1	5.081*
	S × T	2	0.098
	S × C	2	0.985
	C × S × T	2	4.051*
	Error	337	
Flower mass fraction	Color (C)	1	2.088**
	Type (T)	1	0.039
	Stage (S)	2	6.997
	C × T	1	3.121
	S × T	2	6.243**
	S × C	2	1.144
	C × S × T	2	0.818
	Error	337	
Stem mass fraction	Color (C)	1	11.789***
	Type (T)	1	1.889
	Stage (S)	2	457.824***
	C × T	1	1.938
	S × T	2	1.595
	S × C	2	1.603
	C × S × T	2	7.629***
	Error	337	
Leaf mass fraction	Color (C)	1	6.029*
	Type (T)	1	0.371
	Stage (S)	2	33.721***

TABLE 1

Continued

	Source	df	F
	C × T	1	1.688
	S × T	2	7.998***
	S × C	2	2.044
	C × S × T	2	0.113
	Error	337	
Total seed mass per flower	Color (C)	1	22.79***
	Type (T)	1	124.92***
	Stage (S)	2	451.92***
	C × T	1	138.38***
	S × T	2	86.96***
	S × C	2	103.18***
	C × S × T	2	41.46***
	Error	288	
Seed number per flower	Color (C)	1	60.355***
	Type (T)	1	0.005
	Stage (S)	2	620.801***
	C × T	1	59.434***
	S × T	2	157.666***
	S × C	2	100.378***
	C × S × T	2	24.468***
	Error	288	
Seed mass	Color (C)	1	6.367*
	Type (T)	1	87.203***
	Stage (S)	2	99.1 ^{23*} *
	C × T	1	30.736***
	S × T	2	11.532***
	S × C	2	3.701*
	C × S × T	2	7.922***
	Error	288	
Fruit set under natural pollination	Color (C)	1	50.690***
	Type (T)	1	931.8 ^{40*} *
	Stage (S)	2	214.399***
	C × T	1	3.706
	S × T	2	206.406***
	S × C	2	20.965***
	C × S × T	2	29.4 ^{65*} *
	Error	48	
Fruit set under hand pollination	Color (C)	1	103.502***
	Type (T)	1	6.098*
	Stage (S)	2	0.039
	C × T	1	21.551***
	S × T	2	2.849
	S × C	2	0.507
	C × S × T	2	1.912
	Error	48	
Seed set under natural pollination	Color (C)	1	47.65***

TABLE 1
Continued

	Source	df	F
	Type (T)	1	39.25***
	Stage (S)	2	652.50***
	C × T	1	77.43***
	S × T	2	207.64***
	S × C	2	153.04***
	C × S × T	2	11.54***
	Error	288	
Seed set under hand pollination	Color (C)	1	1.655
	Type (T)	1	49.398***
	Stage (S)	2	864.073***
	C × T	1	58.837***
	S × T	2	2.419
	S × C	2	11.324***
	C × S × T	2	2.871
	Error	288	

***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Plant growth is often limited by low temperatures during early spring in our study site. Nevertheless, early emerging plants like those of *G. leucomelaena* tend to undergo a longer growing period before flowering (because of the effects of low temperature on the timing of flowering) such that early emerging plants tend to accumulate greater biomass and grow to a larger size compared to plants that

emerge later during the flowering season. These tendencies help to explain why the shoots of early-flowering plants tend to be more massive and taller than those of later flowering plants (Houle, 2002). Larger plants usually produce more and/or larger seeds than smaller plants (Zimmerman and Aide, 1989; Elle and Carney, 2003; Guo et al., 2010). In the case of *G. leucomelaena*, larger plants reproducing in the early flowering season do in fact produce larger but not more seeds. Our artificial pollination experiments clearly show that pollen transfer is necessary for improving seed yield in all flower types with the statistical exception of the white protogynous (self-pollinating) morph during the early flowering season. Consistent with these observations, fruit set and seed set are lower for early-flowering shoots than for all of the other shoots, and pollinators were largely absent in the early flowering stage. These observations collectively indicate a pollinator and pollen limitation during the early flowering stage (Fig. 5).

G. leucomelaena appears to have evolved two different strategies for coping with a shortage of pollinators during the early spring growing season. One strategy is to produce more white flowers on early-flowering shoots. We have shown previously that the white flowers of *G. leucomelaena* are more likely to be self-pollinated than blue flowers, which are characteristically insect pollinated (Mu et al., 2011), and that white flowers usually have higher anther temperatures, which facilitate pollen development during the early flowering season (Mu et al., 2010). The other strategy is to produce more protogynous flowers on early-flowering shoots. Protogyny has been interpreted to be an adaptation that forestalls selfing when pollinators are rare or absent (Webb, 1981). The production of more protogynous flowers early in the season collectively may enhance seed yield. We did observe that white and protogynous flowers had higher fruit and seed set during the early flowering season compared to blue and protandrous flowers, although the latter might help to avoid autonomous selfing (Mallick, 2001). In addition, flowers produced early in the season might have a

TABLE 2

Biomass allocation, shoot height and reproductive efficiency at three flowering stages for four types of flowers of *G. leucomelaena*.

Flowering stage	Dichogamy	Color	n	Biomass (mg)	Shoot height (cm)	Flower mass fraction	Stem mass fraction	Leaf mass fraction	Reproductive efficiency (N)
Early flowering	G	B	30	10.780 ± 0.643 ^{def}	1.16 ± 0.27 ^a	0.689 ± 0.014 ^a	0.024 ± 0.0003 ^a	0.288 ± 0.0027 ^{bc}	0.042 (30)
	G	W	30	10.474 ± 0.432 ^{de}	1.01 ± 0.19 ^a	0.695 ± 0.016 ^a	0.016 ± 0.0002 ^a	0.289 ± 0.0029 ^{bc}	0.407 (25)
	T	B	30	11.795 ± 0.554 ^{ef}	1.52 ± 0.25 ^a	0.663 ± 0.017 ^a	0.018 ± 0.0002 ^a	0.318 ± 0.0031 ^c	0.020 (30)
	T	W	30	12.497 ± 0.448 ^f	1.37 ± 0.23 ^a	0.656 ± 0.015 ^a	0.017 ± 0.0002 ^a	0.328 ± 0.0029 ^c	0.038 (30)
Middle flowering	G	B	30	9.388 ± 0.431 ^{cd}	2.28 ± 0.17 ^b	0.698 ± 0.012 ^a	0.037 ± 0.0004 ^{ab}	0.265 ± 0.0025 ^{bc}	0.240 (30)
	G	W	30	7.916 ± 0.430 ^{bc}	2.30 ± 0.11 ^b	0.697 ± 0.010 ^a	0.039 ± 0.0004 ^{ab}	0.264 ± 0.0020 ^{bc}	0.316 (30)
	T	B	30	10.806 ± 0.499 ^{def}	2.78 ± 0.39 ^b	0.692 ± 0.009 ^a	0.034 ± 0.0005 ^{ab}	0.274 ± 0.0018 ^{bc}	0.484 (30)
	T	W	30	9.341 ± 0.365 ^{cd}	2.93 ± 0.49 ^b	0.704 ± 0.011 ^a	0.048 ± 0.0005 ^b	0.248 ± 0.0022 ^b	0.206 (30)
Late flowering	G	B	30	5.216 ± 0.236 ^a	5.08 ± 0.32 ^c	0.629 ± 0.013 ^a	0.117 ± 0.0015 ^d	0.249 ± 0.0023 ^b	0.144 (30)
	G	W	30	6.283 ± 0.252 ^{ab}	5.28 ± 0.36 ^c	0.635 ± 0.012 ^a	0.087 ± 0.0009 ^c	0.275 ± 0.0026 ^{bc}	0.099 (30)
	T	B	30	6.757 ± 0.251 ^{ab}	5.65 ± 0.28 ^c	0.700 ± 0.012 ^a	0.110 ± 0.0015 ^{cd}	0.187 ± 0.0024 ^a	0.086 (30)
	T	W	30	7.598 ± 0.231 ^{bc}	6.03 ± 0.28 ^c	0.657 ± 0.014 ^a	0.112 ± 0.0011 ^{cd}	0.233 ± 0.0026 ^{ab}	0.077 (25)

G: Protogyny, T: Protandry, B: Blue, W: White. Values followed by the same letter in the same column are not significantly different at the $P < 0.05$ level according to analyses of variance.

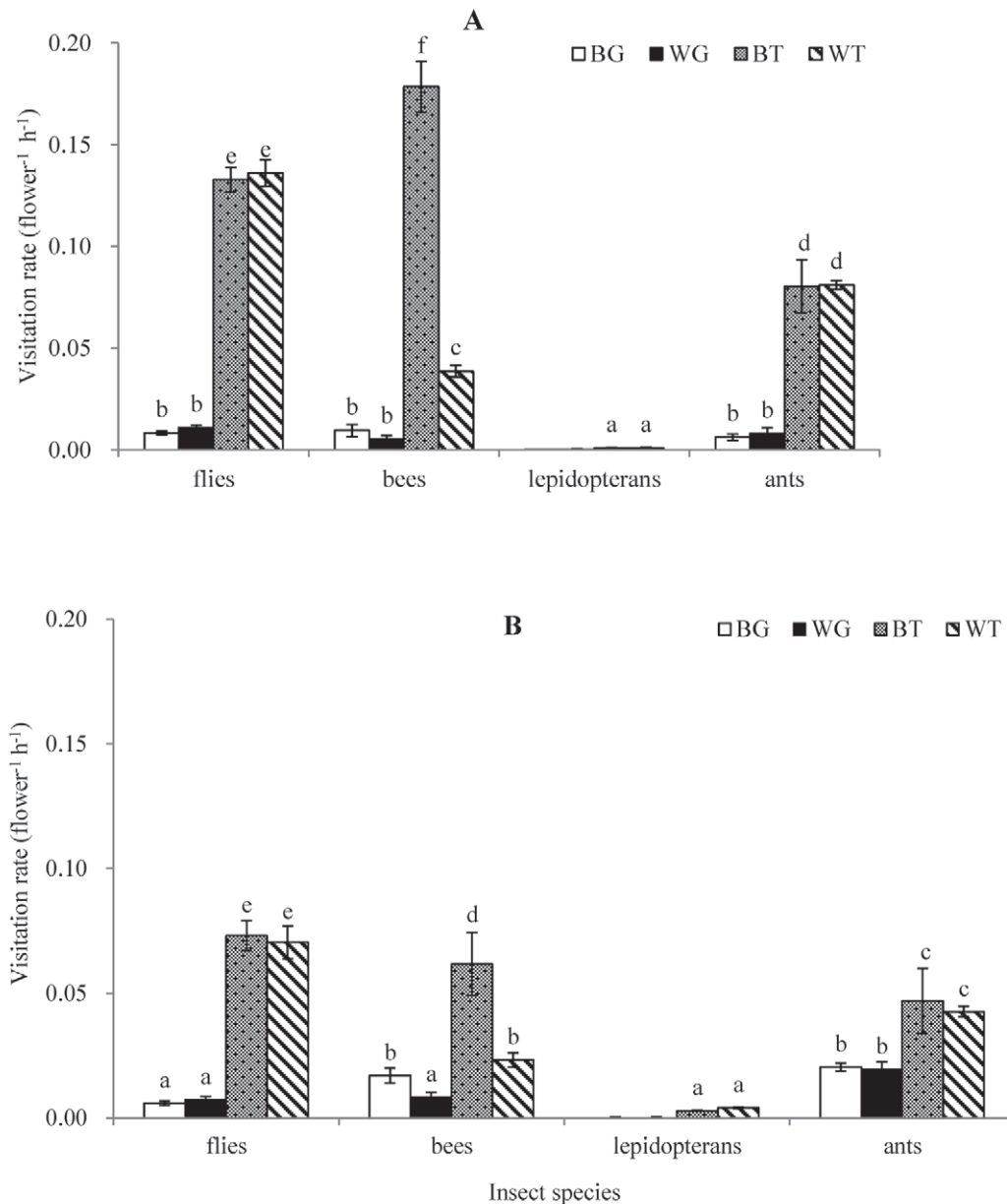


FIGURE 2. Means (± 1 SE) pollinator visitation rates for four flower types [blue and protogynous flowers (BG), white and protogynous flowers (WG), blue and protandrous flowers (BT), and white and protandrous flowers (WT)] at the (A) middle and (B) late stages of the flowering period. The means are daily averages, which were calculated from hourly observations. $n = 5$ observations per day for each flower type.

longer time to develop seeds and thus produce larger seeds (flowers produced early in the season are often observed to have fruits mature at about the same time as flowers produced late in the flowering season; data not shown).

Despite the differences in aboveground biomass, insect visitation rates, and the biomass proportionalities of the four different types of flowers, early- and late-flowering shoots have similar reproductive efficiencies primarily because of low levels of seed number. The reduction in shoot size during the late flowering stage appears to be the result of competition for light and space among plants. Although our study species produces shoots that emerge earlier in a dense meadow than many other grass and forb species (see Liu et al., 2011) that mostly emerge and grow from late April and early May, the maximum height of *G. leucomelaena* is very short and plants are

gradually shaded by neighboring species that tend to be members of much taller species. A similar temporal pattern of plant height growth has been reported in an old-field grassland in the eastern United States (Sun and Frelich, 2011). In this respect, late flowering shoots are generally taller but have thinner stems (as indicated by taller plant height but smaller aboveground plant biomass), which is a typical growth response to shading stress (Ida et al., 2012). We are sensitive to the possibility that late-flowering shoots flower precociously when they have smaller plant biomass because of an intrinsic senescence response to early reproduction and to environmental cues such as increased temperature and photoperiod in late spring (e.g., Sawada et al., 1997; Houle, 2002; Badri et al., 2007). If so, the attending low seed production, as well as the small seeds, could be simply the result of smaller aboveground biomass

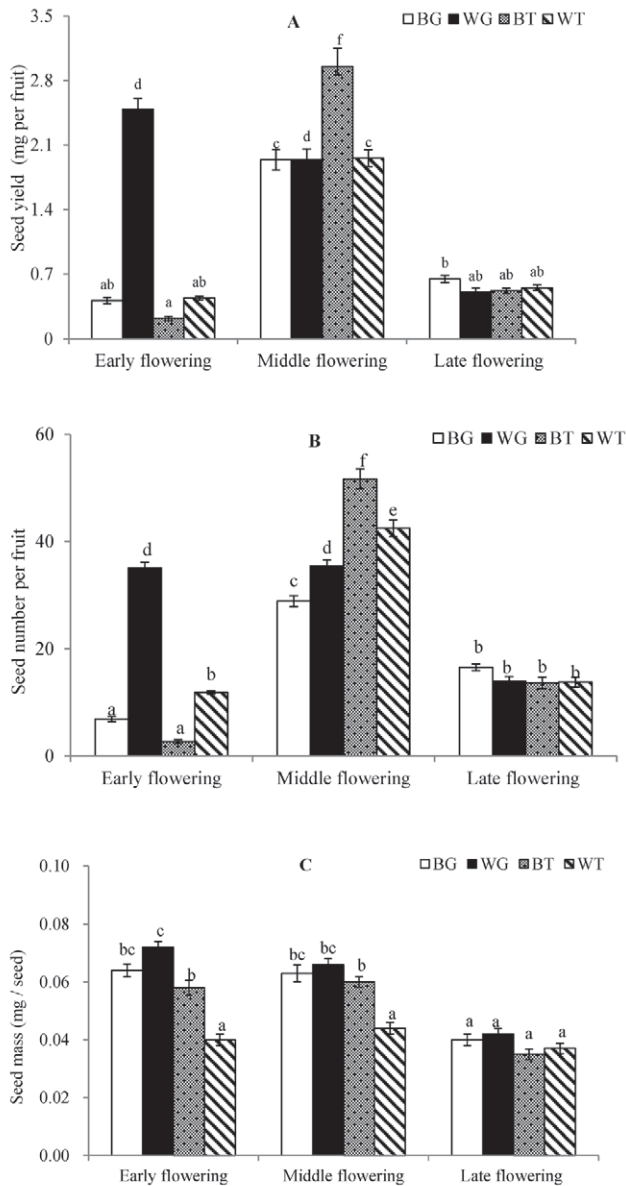


FIGURE 3. Means (± 1 SE) of (A) seed yield (total seed mass), (B) seed number, and (C) seed mass for each of the four types of flowers [blue and protogynous flowers (BG), white and protogynous flowers (WG), blue and protandrous flowers (BT), and white and protandrous flowers (WT)] during the flowering season. $n = 30$ for each flower morph. The different letters above the error bars denote significant difference ($P < 0.05$) among the four flower types.

during the late flowering stage. The small seeds could also be due to a short time for seed development. Finally, it is worth noting that late flowering shoots were reproductively unresponsive to hand-pollination, although their fruit set and seed set were lower than that of middle flowering shoots. This further suggests to us that plant biomass, rather than pollination, limits the seed production in the late flowering stage. Thus, in contrast to pollination limitation occurring during the early flowering season, we believe our observations are collectively consistent with a resource-limitation hypothesis during the latter part of the flowering season when pollinators are comparatively abundant.

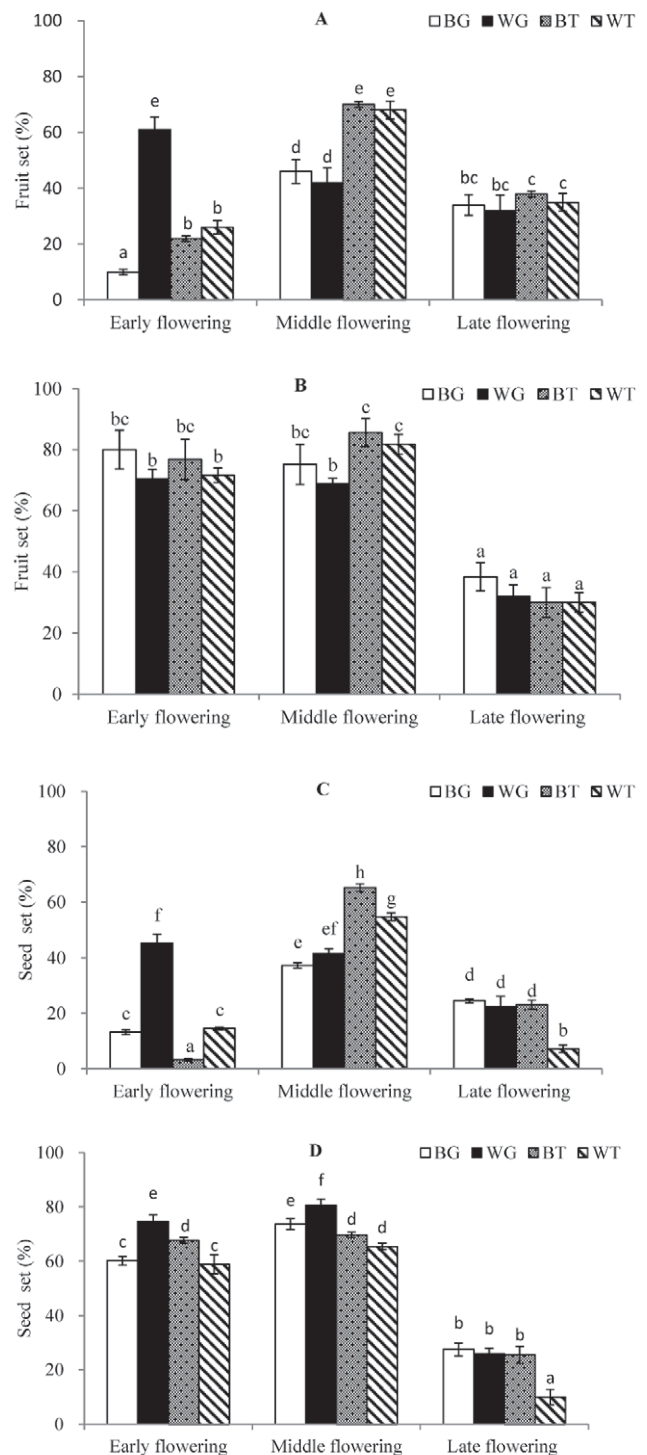


FIGURE 4. Fruit set (%) following (A) natural pollination and (B) hand pollination; and seed set (%) following (C) natural pollination and (D) hand pollination for each of the four types of flowers [blue and protogynous flowers (BG), white and protogynous flowers (WG), blue and protandrous flowers (BT), and white and protandrous flowers (WT)] during the flowering season. The different letters above the error bars denote significance difference ($P < 0.05$) between flower types or stages. $n = 17$ for fruit set in the early flowering stage, and $n = 20$ in both middle and late flowering stages. $n = 30$ for seed set except for the white protogynous flower morphs in early flowering stage ($n = 25$) and white protandrous flower morphs in later stage ($n = 25$).

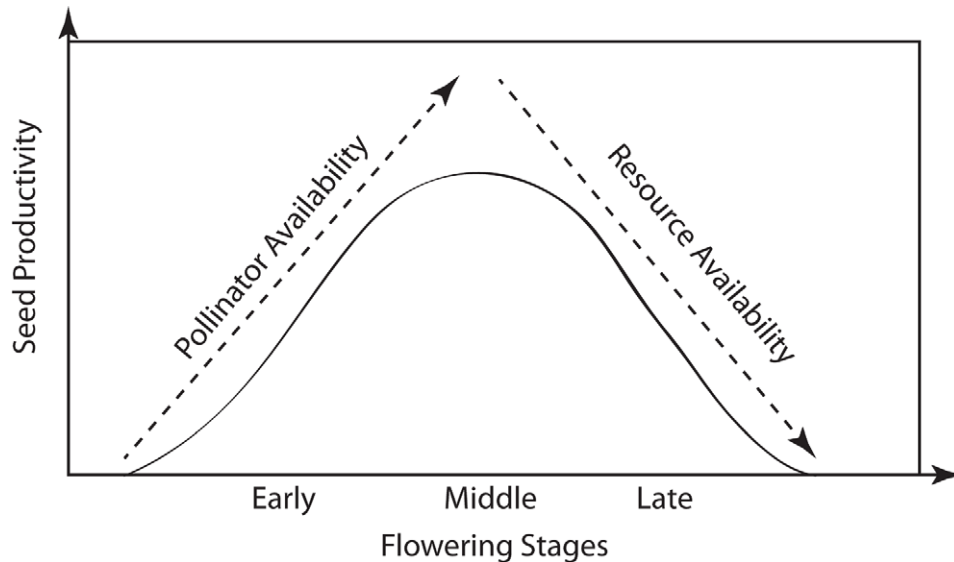


FIGURE 5. A schematic of the interrelationships among seed productivity, pollinator and resource availabilities, and the three stages in the flowering season (early, middle, and late; see Fig. 1). Seed productivity is a function of the availability of pollinators (insects) and resources (e.g., light and soil nutrients). The availability of pollinators increases from the early- to the late-flowering stage; the availability of resources decreases from the early- to the late-flowering stage. These two opposing trends result in maximum seed productivity during the middle-flowering stage, but permit limited seed yield across the entire flowering season.

It is worth noting that a low seed yield during the early and late stages of the flowering season is not necessarily an evolutionary disadvantage. *G. leucomelaena* lives in an alpine meadow characterized by dramatically fluctuating climates. Temperature and rainfall often vary considerably during early spring, and deep frosts can occur after spring warming. Despite their low seed yield, plants that flower early produce large seeds that might have a higher establishment success and that might give rise to seedlings with a greater resistance to fluctuating environments or harsh climatic conditions (Pluess et al., 2005). By the same token, the smaller seeds produced by shoots flowering late in the season may contribute to wider seed dispersal and thus population expansion.

In summary, we have shown that there are two different kinds of limitations to seed yield that change in their intensity over the course of the growing season of *G. leucomelaena*. We further show that this species has two different strategies for coping with these limitations that involve four different types of flowers. Nevertheless, it is worth noting that our conclusion is primarily based on field investigations with randomly sampled shoots; we did not directly test whether low seed production is due to pollination limitation or resource limitation by performing experiments examining the effects of plant biomass and pollinator density on seed production in the species at each flowering stage. Therefore, more research is needed to confirm the mechanisms underlying seed production pattern for this spring ephemeral species.

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