Abstract:
Knowledge about the factors restricting reproduction in many rare wetland plant species is limited. One such species is Lobelia boykinii. We conducted a study of L. boykinii to determine which factors most influence its reproductive success. We examined the species’ breeding system, population dynamics, degree of pollen limitation, importance of sexual versus asexual reproduction, and the role of fungal and herbivore damage on fruit production in three Carolina bay populations in North Carolina. Pollen manipulations revealed that L. boykinii is an obligate outcrosser and that sexual reproduction is pollen-limited. Population size and density, which influence pollinator attraction, can fluctuate greatly over years in the same population. The three populations differed with respects to population size and density, capsule production, vegetative reproduction, and ramet survival, and differences are partially attributable to variation in the degree and timing of herbivory and fungal damage. Herbivory ranged from < 1% to 55% of flowering shoots. Fungal infection decreased fruit production only when it coincided with the period of floral development. Our results indicate that focusing on one ecological factor to prevent extant populations from going extinct is insufficient. Multiple intrinsic and extrinsic factors influence L. boykinii's reproductive success, and populations differ in which factors most strongly influence reproduction. Given this suite of constraints, management strategies should include maintaining as many of the largest remnant populations as possible.

Key words: breeding system, Carolina bays, demography, endemic, flowering phenology, fungal infection, Lobelia boykinii, pollen limitation, rare plant.
to some of these sites indicate that this site number has dropped (Lacey, unpubl. data). Presently, the species has a G2/G3 global rank indicating a globally imperiled or rare species (NatureServe 2006). The species is listed as endangered in Florida and New Jersey and is under consideration for federal listing (USDA, NCRS, 2006).

In a previous study, we examined the effects of hydrologic fluctuations on population persistence (Lacey et al. 2001). In this study, we examine aspects of the species' biology that may independently or in concert with additional ecological factors contribute to the rarity of Lobelia boykinii and more specifically affect reproductive success. We examine its breeding system, flowering phenology, reproductive output, and whether or not factors such as pollinator availability, herbivory, and fungal infection limit reproductive output. Also, we explore its growth and survivorship, which affects population size and density, and in turn influence pollinator visitation rate. Knowledge of L. boykinii's reproductive biology and constraints will help managers develop better maintenance and restoration plans for this and other rare wetland species that possess similar rosette life-forms and habitat restrictions.

**BIOLOGY OF LOBELIA BOYKINII**

The life cycle and geographic distribution of Lobelia boykinii have been described elsewhere (Lacey et al. 2001), but we summarize here features relevant to this study. Seeds germinate during August and early September and seedlings develop into rosettes during the fall and grow throughout the winter. In April, rosettes disappear from aboveground view to reappear the next fall, when they recommence aboveground growth, and presumably also belowground growth. If a rosette survives its first nineteen months of life, it either bolts and flowers or again dies back to the rhizome in the second spring. If an individual rhizome survives after flowering, it produces one to several new rosettes the following fall. Due to the ephemeral nature of rosettes and flowering shoots, population size can be estimated only in winter, when both seedlings and established adult rosettes are visible. In this paper, the word plant refers to a ramet, which is either a rosette or flowering shoot.

Bolting plants flower in June-July, and mature capsules dehisce throughout July-August (Bates 1996). In Lobelia boykinii, the elongating style of a flower pushes the pollen out of the anther tube and only then opens to expose the receptive and pollen-free stigmatic surface for pollination. The stamens, stigmas, and capsules of some flowers on some plants are infected by fungi. At times, the fungal infection completely envelops the anther tube and prevents stigma exertion.

**STUDY SITES**

Our data, collected from 1994 to 1998, come from populations growing in three Carolina bays (hereafter referred to as 'bays') in North Carolina: Antioch Church Bay (ACB) in Hoke County and Big Cypress Meadow (BCM) and State Line Prairie (SLP) in Scotland County. These bays are owned by The Nature Conservancy and are three of the five bays in which Lobelia boykinii is known to grow in North Carolina. The other two are located adjacent to or on military bombing practice sites.

The study bays differ in their size, hydrology, degree of vegetation openness, and depth of accumulated litter (Royo 1998). ACB, although intermediate in size (34.8 ha), has the greatest concavity and typically holds far more water than the other two bays (Royo 1998). ACB is characterized by an open canopy of pond cypress (Taxodium distichum var. nutans (Ait.)) and a very sparse herbaceous layer. SLP (12.14 ha) and BCM (54.6 ha) are much drier, and one cannot predict which bay would hold more water in any particular season (Royo 1998, Lacey et al. 2001). SLP lacks a mature tree canopy although the ground layer contains young loblolly pine saplings, a well developed herbaceous layer, and little accumulated litter. In BCM, both loblolly pine (Pinus taeda) and pond cypress dominate in the open canopy, and the ground layer is characterized by abundant herbs and a thick carpet (sometimes greater than 5 cm) of pine needles. All bays had been affected by infrequent wildfires, although precise information is lacking (Bücher, North Carolina Chapter of The Nature Conservancy (NCTNC, pers. comm.). At the time of our study, the last recorded fire in ACB had occurred in 1991 and in 1988 in both SLP and BCM.

**Methods and Materials**
Demographic Data. Germination was estimated from randomly established experimental plots (0.5 X 0.5 m) established within each population in 1995 and 1997. Plots were located in patches of bare ground in the herbaceous cover. Soil in these patches was saturated but not covered with standing water in 1995. In late July-early August of each year, seeds were collected from multiple plants in each bay. In late August early September 1995, we scattered 100 fresh seeds onto each of seven plots in ACB, 10 in BCM, and seven in SLP. Forty-nine seeds were scattered onto an eighth plot in ACB. In 1997, we scattered 100 seeds onto each of two, five, and four plots in ACB, BCM, and SLP, respectively. Experimental plots were paired with adjacent controls and monitored through the fall for germination. Germination was calculated as the difference in seedlings numbers between the seed addition plots and the paired controls.

Seedling survival was estimated from naturally germinated seedlings. In September 1994, we found a total of 321 naturally germinated seedlings in ACB and SLP, and in 1995 we found a total of 95 seedlings at all populations. From this sample of marked seedlings, we determined the proportion that survived until the following January.

Collecting data on survival and vegetative propagation of adult plants is challenging because rosettes, which one can easily count, live at most for one year, but a rhizome, the perennating organ, may persist for many years. We estimated rhizome survival by monitoring the fate of adult rosettes marked during September and October 1994 (63, 60, and 132 in ACB, BCM, and SLP, respectively). Adult rosettes are produced by rhizomes, and through October these rosettes are easily distinguished from seedlings and new rosettes, which still have cotyledons. Our measure of survival was the proportion of rhizomes that had an adult rosette in fall 1994 and that produced a new rosette in fall/winter 1995. If an adult in 1994 was succeeded by only one rosette in fall 1995, we recorded that the rhizome survived. If an adult rosette was succeeded by more than one rosette in fall 1995, we recorded that the rhizome had propagated itself vegetatively.

For surviving rhizomes, we recorded the number of new rosettes and measured the distance between parent and offspring rosettes to calculate clonal spread. Based upon the growth patterns of single rhizomes grown in pots in the greenhouse (see Bates 1996), we assumed that a new rosette found within 5 cm of the previous year's rosette was propagated asexually from the surviving rhizome. Any rosettes that could not be confidently assigned to a previous year's rosette (e.g., because of proximity to more than one previous year's rosette) was eliminated from the dataset. Trying to trace intact rhizome growth in situ was difficult because roots of other species are often intertwined with the already fragile Lobelia boykinii rhizome, whose diameter is typically ≤ 0.5 cm (Lacey, pers. obs.).

Estimates of population size and density were conducted in winters 1995-96 and 1997-98. In each year we surveyed each bay to locate the Lobelia boykinii populations and delineated each population within a rectangular area to determine population spatial extent. Within these areas we randomly established 1 m2 vegetation sampling plots and recorded rosette density. We then extrapolated plot density to the total area encompassing the known population to obtain an estimate of total population size (number of individuals in entire population). In 1996 we sampled 45, 59, and 76 plots within ACB, SLP, and BCM, respectively. In 1998 we sampled 36, 33, and 47 plots within ACB, SLP, and BCM, respectively.

We also documented herbivory of flowering shoots in summers 1995 and 1996. In January 1995, we augmented the number of rosettes that we had marked in October 1994. Those that subsequently bolted were used to document herbivory (sample size = 142, 164, 329 for ACB, BCM, SLP, respectively). In fall 1995, we marked 100 rosettes per population and recorded herbivory on those that bolted in summer 1996. Herbivory was estimated as the proportion of shoots that had evidence of herbivore damage, regardless of whether or not a shoot produced a subsequent, unbrowsed inflorescence.

We used generalized linear models to test for differences among populations in percent germination, seedling and adult rosette survival, vegetative reproductive output, and percent herbivory. When a significant F value
was detected, least significance difference (Tukey LSD) was used to compare means among populations. Seed germination was strongly skewed towards zero and was best modeled using a Poisson distribution and vegetative reproductive output was modeled using a normal distribution. All other data were modeled assuming a Beta distribution.

**BREEDING SYSTEM**

During 1994 and 1995 we identified the type of breeding system of Lobelia boykinii by applying five manipulations of the pollination syndrome to individual flowers on single plants and observing capsule development. In 1994, autogamous (i.e., self-fertilized) flowers were caged with fine-mesh mosquito netting prior to anthesis and left undisturbed throughout the flowering season. Geitonogamous (pollinated from different flowers on same individual) flowers were caged before anthesis. Once their stigmas became receptive, we hand-pollinated caged flowers with pollen from another flower on the same inflorescence. Apomictic (i.e., without pollination) flowers were caged after excising their anthers. Xenogamous (i.e., cross-pollinated) flowers were hand-pollinating with pollen from different plants. Control flowers were uncaged and left to be pollinated naturally. Each flower pollination treatment was randomly applied to one of five flowers on 32 plants in ACB. We set up only the control and autogamy treatments on 15 plants in SLP because fewer flowering plants, with fewer flowers per plant, were present in this bay. We could not use the BCM population for the breeding system experiment due to the small population size (Bates 1996).

In 1995 we conducted a second experiment on 22 plants in ACB to test the possibility that the presence of cages around individual flowers may have influenced the 1994 experimental results. For this experiment, we caged whole inflorescences in the bud stage and hand-pollinated only some flowers in each cage using pollen from a different individual. Hand-pollinated flowers alternated with unpollinated flowers on a shoot to reduce position effects. In both experiments, our dependent variable was the proportion of treated (or untreated) flowers per plant that produced capsules.

**REPRODUCTIVE OUTPUT AND FLORAL VISITORS**

Reproductive output per plant was measured in summer 1995 for all three populations. To assess if pollen/pollinator availability limited reproductive output we compared capsule production success (i.e., proportion of marked flowers that set fruit) and seeds per capsule in flowers that were naturally-pollinated or hand-pollinated (augmenting the pollen load) in the three populations in 1995. We used 27, 20, and 21 flowering plants scattered from the edge to the center of each population in ACB, BCM, and SLP, respectively. Total flower number per plant varied among individuals and thus, number of flowers per treatment (control vs. hand-pollinated) varied among plants. For most plants, flower number between treatments was balanced (Bates 1996). Stigmas of hand-pollinated flowers received pollen from another individual at least three meters away. Naturally-pollinated flowers, which alternated with hand-pollinated ones, were not manipulated. Mature capsules were harvested as they ripened, and the proportion of marked flowers producing capsules and seed number per capsule was recorded for each treatment by plant combination.

We used generalized linear mixed models (PROC GLIMMIX, SAS/STAT 2004) to test the effect of population and treatment on seed number per capsule and capsule production. Population and Treatment (control vs. hand-pollinated) were treated as fixed effects, and Plant was treated as a random variable. When a significant F value was detected, least significance difference (Tukey LSD) was used to compare treatment means. When data exhibited large deviations from normality we analyzed the data by fitting the most appropriate distribution. Capsule production exhibited a U-shaped distribution and was modeled with a Beta structure. This latter distribution is particularly well suited to variables that exhibit strong skew and are bounded by two known endpoints (e.g., proportional data; see Smithson and Verkuilen 2006).

When we visited the populations to do field work during the flowering season, we looked for floral visitors (many days in 1994 and every day in 1995). However, our searches were not systematic because it soon became obvious that observing floral visitors in individual plants for a fixed observation period was inefficient due to the paucity of recorded visits. Instead, we collected representative individuals of all insect visitors that we
observed on Lobelia boykinii flowers from 8:30 am to 5:00 pm. Using a dissecting microscope we looked for L. boykinii pollen on these insects. All specimens bearing L. boykinii pollen were identified to species by the North Carolina Department of Agriculture.

**FLORAL AND FUNGAL PHENOLOGY**
We monitored the phenology of flowering, fruiting, and infection on a sample of three flowers on each of 50 plants in each population. A flowering shoot produces an indeterminate inflorescence, with flowers maturing from the bottom to the tip. It is not uncommon for a shoot to produce 10 or more flowers. At the beginning of the flowering season, we divided the inflorescence into three positions: low-level flower (1st-5th positions), mid-level flowers (6th-11th position), and high-level flower (≥ 12th position). Plants that produced six or fewer flowers were not used in the analysis, which reduced the sample size by one plant in SLP and 15 in BCM. Data on fungal presence and successful (mature) capsule production were recorded for each marked flower through the flowering season in 1995. Samples of infected flowers were sent to the Plant Disease and Insect Clinic at North Carolina State University for fungal identification.

For our analysis, we restricted the dataset to only those reproductive units that had completed flowering by June 26, 1995. First, we created a reproductive outcome variable with four states: capsule present/fungus absent, capsule and fungus present, capsule absent/fungus absent, capsule absent/fungus present. We then employed a split-plot analysis using a baseline-category logit model to assess whether the proportions of four reproductive outcomes varied across populations and over the three floral positions (e.g., phenological stages). Plant was treated as a random variable nested within population.

**Results**

**DEMOGRAPHIC DATA AND HERBIVORY**
Seed germination was very low. Ramet density varied dramatically across populations and over time. Population density in ACB dropped from 6.2 ± 1.3 ramets/m² in 1996 to 0.3 ± 0.1 in 1998. In SLP the population dropped from 8.0 ± 2.7 ramets/m² in 1996 to 2.42 ± 1.2 in 1998. Based on plot censuses alone, we estimated the BCM population to be near zero in both years, although after an exhaustive survey of the area in 1998 we managed to locate 28 individuals resulting in an estimated density of 0.02 ramets/m². Estimated spatial extent of the three populations contracted from 16,800 m² to 3584 m² in ACB, 3819 m² to 1425 m² in SL P, and 5427 m² to 1215 m² in BCM. Estimated population size within bays between winter 1996 and winter 1998 suggested a 99% decline in ACB (104,832 individuals in 1996 vs. 896 individuals in 1998) and an 89% decline in SLP (31,383 individuals in 1996 vs. 3454 individuals in 1998).

Herbivory of Lobelia boykinii shoots significantly differed among populations in 1995 ($F_{2,197} = 17.4$, $P < 0.001$). Populations also differed in their response to an early (May 1995) grazing. In SL P, 28% of the grazed plants successfully fruited from a secondary flowering stalk. In contrast, no grazed shoot in BCM subsequently fruited. Lack of herbivory in ACB in 1995 precluded this assessment. A variety of large (e.g., Odocoileus virginianus) and small (e.g., Microtus spp.) mammals as well as insects (e.g., grasshoppers; Orthoptera: Caelifera) would likely be agents of herbivory at these sites. Nevertheless, the 45° angle cut on clipped shoots is characteristic of damage by rabbits (Rezendes, 1992).

**BREEDING SYSTEM**
In both 1994 and 1995, capsules were produced only in the control and xenogamy treatments. In 1994 in ACB, the proportion of flowers producing capsules in the control and xenogamy treatments were $0.69 ± 0.08$ (SE) and $0.97 ± 0.02$, respectively. In 1995 in ACB, only hand-pollinated flowers within enclosed inflorescences fruited and the proportion capsule production was $0.81 ± 0.16$. In 1994 in SLP, the proportion of capsule production was $0.4 ± 0.13$ (control treatment). No flower in the autogamy, geitonogamy, or apomixis treatments produced a capsule.
REPRODUCTIVE OUTPUT AND FLORAL VISITORS

The three populations differed significantly in capsule production success although not in seed number per capsule (main population effect on capsule number: $F_{2, 65}^\text{sub} = 5.10, P = 0.009$; main effect on seeds per capsule: $F_{2, 63}^\text{sub} = 2.89, P = 0.063$). The probability that a flower would develop a capsule was significantly higher for ACB than for the other populations (ACB = 83%, BCM = 60%, SLP = 63.5%; Table 1). Hand-pollination significantly increased percent capsule production by 31% and seed number per capsule by 52% (main effect on capsule number: $F_{1, 65}^\text{sub} = 11.0, P = 0.0015$; main effect on seeds per capsule $F_{1, 63}^\text{sub} = 14.2, P = 0.0004$; Table 1).

We observed a total of seven insect species visiting Lobelia boykinii flowers in ACB and in BCM, but none in SLP. Most visitors were Hymenopterans, and all but one carried pollen. They were: Ceratina dupla (Hymenoptera: Apoidea), Augochlorella striata (Hymenoptera: Halictidae), Hylaeus confluens (Hymenoptera: Apoidea), Hylaeus modestus (Hymenoptera: Apoidea), Hoplitis truncata (Hymenoptera: Apoidea), Melissodes communis (Hymenoptera: Apoidea), and Geron holosericeus (Diptera: Bombyliidae). For no species did we observe more than a handful of visitation events; exact numbers were not recorded. Ceratina dupla and Augochlorella striata have previously been reported to visit Lobelia spp. (Mitchell 1962). We observed no pollen on Pieris rapae (Cabbage butterfly) and therefore considered it an insect visitor.

FLORAL AND FUNGAL PHENOLOGY

Floral development in Lobelia boykinii differed among the three populations (Fig. 3). Individuals in ACB and SLP began flowering and fruiting earlier than did those in BCM, and the ACB population finished most quickly. Sixty-four percent of the flowers in BCM were still in the bud stage on June 1 in contrast to 44% and 46% in ACB and SLP, respectively. All but 4% of the flowers in ACB had finished flowering by June 26 compared to 14% and 23% in SLP and BCM, respectively.

The NCSU Plant Disease and Insect clinic identified the fungi found on flowers and fruits as Botrytis cinerea and Alternaria spp. Fungal infection increased throughout the flowering season although the timing and intensity of fungal infection differed slightly among the populations (Fig. 3). Fungi appeared first in SLP but increased most quickly in ACB as evidenced by the sharp rise from June 1-13. The effect of fungal infection on capsule production differed among populations and floral position (Population × Floral Position interaction: $F_{12, 976}^\text{sub} = L8, df = 12, P = 0.046$). Fungal infection increased capsule production in ACB on the low floral position, lowered capsule production in BCM on the low floral position, and had no impact on capsule production in SLP on any floral position (Fig. 4). Infection was most severe in ACB with approximately 90% of the flowers infected by fungus by the end of June and least severe in SLP with approximately 64% of flowers infected.

Discussion

The combined data provide strong evidence that the reproductive success in Lobelia boykinii is limited by multiple interacting factors that include breeding system, pollinator limitation, herbivory, population size and density, and fungal infection. Data also indicate that the negative impact of these factors varies across populations and years.

First, our experiments show that Lobelia boykinii is an obligate outcrosser. This type of breeding system likely limits seed production in at least some populations because of small population sizes. Small populations typically have a reduced number of genotypes available for cross-pollination (reviewed by Ellstrand and Elam 1993). Also, inbreeding depression effects (e.g., lower seed set, reduced germination) are typically more severe in small populations of self-incompatible species, such as L. boykinii, than in equally sized self-compatible species (Barrett and Kohn 1991, Huenneke 1991, Menges 1991, DeMauro 1993). The very low germination in our experimental plots might be partially explained by inbreeding depression, although this needs testing.

Obligate outcrossing can negatively affect reproductive success also in clonal species when pollinators forage among close neighbors. Such a foraging pattern results in most of the pollen being transferred within genotypes,
rather than between genotypes (e.g., Huenneke 1991, Charpentier 2002). Our data suggest, however, that clonal growth in Lobelia boykinii is not likely to influence pollen transfer because few ramets, flowering shoots, are produced each year from a single rhizome.

Seed production in our study populations appears to be pollen-limited because of low pollinator visitation rates. Hand-pollinations, which augmented the pollen received by stigmas, significantly increased capsule production and/or seeds per capsule. Lobelia boykinii appears to rely on floral visitors for successful pollination. However, our counts of floral visitors were very low. Mechanistically, pollen limitation may result from decreased pollinator abundance and/or decreased pollinator attraction. Our study populations were embedded in an agricultural matrix that was being regularly sprayed with insecticides, which may have reduced pollinator populations. Alternatively or additionally, pollen limitation may have resulted from small population size or low population density, either of which can affect pollinator attraction (reviewed by Knight et al. 2005). Plant density is the element more closely linked to decreased pollinator visitation and pollen transfer rates in other species (Sih and Baltus 1987, Kunin 1997, Mustajärvi et al. 2001, Knight 2003).

All study populations showed a dramatic collapse in density and size from 1996 to 1998. Although we did not measure population size and density in 1995, when we collected our data showing that pollen was limited, casual observations suggested that population size and density changed little from 1995 to 1996 in our study bays. Thus, it was at the higher population size and density, 1995 vs. 1998, that we observed pollen limitation. As an aside, in 2001 the population size in ACB was much larger than in 1996 because a 5-year drought allowed that population to expand from the periphery into the center of the bay, which in previous years had very deep standing water (Moreno 2003). Thus, it is clear that population size and density can vary dramatically across years for Lobelia boykinii. In most years, size and density probably affect pollen transfer.

Our data also show that herbivores and pathogens can lower reproductive success. For both the ACB and SLP populations, herbivory of flowering shoots was higher in 1996, when plants flowered in no standing water, than in 1995 when plants flowered in at least 5 cm of standing water (Royo 1998). Herbivory was very low.

The seasonal timing of herbivory and fungal infection relative to the timing of floral and fruit development appears to influence how strongly each reduces seed production. Even though the SLP population suffered greater herbivory than did the BCM population, the damage occurred early in the flowering season, and many plants had enough time to produce a second inflorescence. BCM shoots flowered later and did not produce secondary inflorescences. Earlier flowering in the ACB and SLP populations probably also contributed to the lower amounts of fungal infection of flowers in these populations. Fungal infection coinciding with floral development is known to interfere with subsequent capsule production in other plant species (e.g., McNicol et al. 1985, Perryman et al. 2002), as was observed in the BCM population. Susceptibility to Botrytis cinerea infection is greatest during early floral development (Perryman et al. 2002). By the time that the incidence of fungal infection was high, in mid-June, floral development was much farther along in ACB and SLP than in BCM.

In conclusion, in order to understand the population dynamics of a rare species, thereby facilitating its survival, one must follow multiple populations over many years (Oostermiejer et al. 1996, Byers and Meagher 1997, Damman and Cain 1998, Menges 2000). However, in conducting such a study, it is desirable to know at the onset what factors influence the species' population dynamics. Our experiments indicate that multiple factors interact to influence the reproductive success, and therefore the population dynamics, of Lobelia boykinii. Long-term monitoring of extant L. boykinii populations should include the monitoring of all these factors. Furthermore, the relative importance of the extrinsic factors, e.g., fungal infection and herbivory, varies both among populations and across years. Given the observed dramatic changes in population size and density across years, management strategies should include preserving as many of the larger remnant populations of Lobelia boykinii as possible.
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