Introduced North American Black Henbane (*Hyoscyamus niger*) Populations are Biennial

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Black henbane can be either annual or biennial. We investigated which life cycle is found in four introduced western North American populations. Plants were grown in a greenhouse common garden until half were vernalized by exposure to natural winter temperatures, while the other half remained in the greenhouse above 20 C, with 16 h of light and 8 h of dark. In total the plants were monitored 313 d after germination. We measured whether plants bolted, the time it took for bolting to commence, and the size at bolting. All vernalized plants bolted after 117 d of active growth (within 26 d of the end of the vernalization treatment), whereas only 26% of the nonvernalized plants bolted after an average of 278 d of active growth. Vernalized plants bolted at a smaller size than the nonvernalized plants that bolted (28 vs. 41 leaves on average). In the nonvernalized plants, the relationship between time to bolting and size was strong, but not so with the vernalized plants. Our results indicate that introduced black henbane plants are biennial, and that vernalization is more critical to bolting and flowering than reaching a certain size. Nonetheless, the fact that nonvernalized plants were capable of bolting if grown long enough suggests that vernalization is not the only cue that can trigger reproduction in introduced populations.

Nomenclature: Black henbane, *Hyoscyamus niger* L. HSYNI.

Key words: Biennial, invasion, vernalization.

The life history strategy of an organism is an important factor in determining individual fitness and population growth rates (Stearns 1976) and, in the case of invasive plants, helps determine whether a species establishes and becomes invasive in a new area (Sol et al. 2012; Sutherland 2004). The length of the life cycle of monocarpic plants is a key life history trait. An annual life cycle may confer an advantage over biennial and perennial life cycles because of the shorter generation time (Baker 1965; Lewontin 1965; Pimm 1991). However, biennial plants often flower at a larger size and therefore typically produce more seed. Thus, there is potentially a trade-off between the cost of a longer generation time and the benefit of higher seed production (Klinkhamer and De Jong 1983; Van der Meijden et al. 1992; Wesselingh et al. 1993).

Both environmental and genetic factors can influence timing of reproduction in monocarpic plants (Johnson 2007; Reinartz 1984). The time to flowering and therefore generation time can be determined by plant size rather than age (Wesselingh et al. 1993). However, often exposure to a particular photoperiod (Parker et al. 1950) or to cold (e.g., vernalization; Bernier et al. 1981) determines a plant's ability to flower. Populations commonly vary, often along latitudinal clines, in the traits that determine timing of reproduction (e.g., prereproductive period, biomass, photoperiod, need for vernalization; Boudry et al. 2002; Quinn 1969; Reinartz 1984). Variation in these traits has frequently been shown to have a genetic basis (Law et al. 1977; Reinartz 1981). More recent studies have revealed a specific "flowering locus," FLOWERING LOCUS C (FLC), in the model organism *Arabidopsis thaliana* (L.) Heynh that is regulated by vernalization and methylation (Finnegan et al. 2005; Michaels and Amasino 2000; Sheldon et al. 2000).

Here we explore two of the factors that influence life cycle length (annual vs. biennial) of black henbane (*Hyoscyamus niger* L.; Solanaceae) in introduced North American populations. Specifically, we examine how plant size and vernalization influence flowering and time to flowering.

Study System. Black henbane, or henbane, is an introduced toxic weed that typically grows in open and disturbed habitats. Henbane is a monocarpic plant with both annual and biennial forms in its native Eurasian range

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Management Implications

Knowledge of the life cycle of an introduced plant is a fundamental component of its successful management. Black henbane (*Hyoscyamus niger*) is a state-listed noxious weed that is toxic to both livestock and humans. There is surprisingly little information on the introduced populations in North America. We explored whether introduced populations follow an annual life cycle, biennial life cycle, or both. We first collected seeds from several naturalized populations. The seeds were germinated, and the resulting plants were grown in a common environment in Fort Collins, CO. We then subjected half of the plants to a winter cold treatment (vernalization), while the other half remained in the greenhouse. All plants sampled appeared to be biennials, with cold being required for timely flowering. This has important implications for the potential of black henbane to spread: it is likely limited to areas that experience at least 10 wk of cold (3 to 11 C) winter temperatures. Combining this information with this species being a poor competitor, requiring open space to thrive, we can infer that it will perform best in fairly open western North American environments with a cold winter. Additionally, given that it is biennial, where active management is necessary in the western United States, monitoring and managing populations over multiple years will likely be key to effective control. Finally, it is critical to guard against the introduction of annual henbane plants to North America. Currently, USDA-APHIS requires a permit to import any part of the black henbane plant or plant products into the United States. Adhering to the current set of national regulations will help limit the range of naturalized black henbane populations.

(Correns 1904; Schläppi 2011; Selleck 1964). The life history in introduced North American populations is currently unknown. Early research on life cycle length in henbane suggests it is determined by a simple dominant allele (Correns 1904). Correns grew seed that had been collected from annual and biennial forms in a common field environment in Europe and found that annuals always produced annuals and biennials always produced biennials. When he crossed the two forms, the F1 offspring were always biennial, whereas the F2 offspring showed segregation, suggesting a single dominant locus determined whether a plant flowered in its first or second year. Further research confirmed that the annual life cycle is recessive (Correns 1904; Lang 1986; Schläppi 2011). Both annual and biennial henbane initiate flowering the first half of the summer season (i.e., they are long-day plants). Recent research on the genetic and physiological basis of flowering in black henbane suggests that there are two types of annuals: late-flowering genotypes that benefit mildly from vernalization and early-flowering genotypes that do not benefit from vernalization (Schläppi 2011). Under a constant temperature (22 C [71.6 F]) and long-day photoperiod, Schläppi (2011) found that early-flowering annuals bolted after 30 to 35 d in pots and flowered at a size of 16 to 18 leaves, whereas late-flowering annuals bolted after approximately 60 d without vernalization and flowered with an average of 26 leaves. Nonvernalized biennial plants in Schläppi's study did not flower, even after 1 yr. Additional studies on vernalization requirements of the biennial form of henbane indicate that at least 10 wk of cold (i.e., 3 to 11 C) are required to trigger flowering (Diomaiuto-Bonnand et al. 1980; Melchers 1937). Thus, if introduced plants flower within 60 d, or shortly thereafter (in a greenhouse with a long-day photoperiod), without vernalization, we can infer that they are annuals, whereas if they do not flower in that time period and respond to vernalization by flowering, we can infer they are biennials.

Table 1. Locations of the black henbane seed collections that were used for this experiment, latitude and longitude of the sites based on GPS coordinates, and number of maternal lines that were collected from each population. Ten siblings were used from each maternal plant.

Location	Latitude	Longitude	Number of maternal lines
Parshall, CO	40°4.129′N	106°15.438′W	5
Rock Springs, WY	41°21.500′N	109°16.165′W	4
Jackson, WY	43°25.386′N	110°46.524′W	11
Cascade, MT	47°14.341′N	111°51.927′W	8

Materials and Methods

Vernalization Experiment. The effect of vernalization on life cycle length of introduced black henbane plants was evaluated by experimentally imposing two temperature treatments (vernalized and nonvernalized) and subsequently measuring whether plants flowered or not, time to flowering, and size at flowering.

Seed was collected from four naturalized introduced populations in the fall of 2009. At each site we collected seed from 4 to 11 maternal plants (see Table 1 for sample sites and sizes). After collection, seeds were removed from their capsules and stored in a refrigerator at 3.4 C until planting. Ten offspring were grown from each maternal plant. Seeds were sown in germination flats with Fafard potting media and placed on a mist bench with a misting regime of 15 s duration every 3 min for 9 h d^{-1} over 12 wk until enough seeds germinated. The median date of germination (here used as the general date of germination) was June 24, 2010, 6 wk after sowing. Seven weeks after germination the seedlings were transplanted to 3.8-L (1-gallon) pots and kept in a greenhouse with a 16/8 h day/night photoperiod and 24.5/17.2 C day/night average temperatures.

Thirteen weeks after germination, on September 24, 2010, we recorded three measures of plant size—the total number of leaves larger than 2 cm, rosette diameter in two orthogonal axes, and the length and width of the largest

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leaf—and then initiated the treatments. Five of the 10 plants from each maternal line were randomly assigned to cold or warm temperature treatments. The plants in the cold treatment were moved outside to experience natural winter temperatures as the vernalization treatment while the other half remained in the greenhouse. Vernalized plants were moved back into the greenhouse after 19 wk outdoors, ensuring that plants experienced at least 10 wk of cold temperatures (3 to 11 C) required for vernalization (Diomaiuto-Bonnand et al. 1980; Melchers 1937). The average daily temperature in Fort Collins, CO, during the period used in our vernalization treatment was 3.89 C (National Weather Service data, accessed on wunderground.com). While outside, vernalized plants were buried in wood mulch and covered with straw. They experienced natural fall and winter weather conditions in Fort Collins and received water by means of snowmelt. Greenhouse plants were watered as needed, typically three times per week. In addition to the plants in the vernalization treatment experiencing colder temperatures than the nonvernalized plants, they also experienced a different light regime. Vernalized plants received low light overall (under mulch, in a lath house), and what light they received had the natural fall and winter photoperiods (short days, long nights). Nonvernalized plants received more light during the treatment period, with the greenhouse lights set to 16/8 h day/night.

After the end of the treatment period all vernalized plants were brought back into the greenhouse. At this point the vernalized and nonvernalized henbane plants once again shared a common garden environment. Days to bolting, days to flowering (with flowering defined as the first day that a flower was open enough to be pollinated), plant size (as described previously), and height (length of the tallest stem) at flowering were measured. Plant size was measured three times after the end of the vernalization treatment: on all plants 1 wk after plants were recombined into the greenhouse (February 12); on all plants 1 wk later (February 19), at which point the majority of the vernalized plants were bolting; and finally, individually as each plant started to bolt. These final measurements spanned 3 m from February 5, 2011, to May 3, 2011, at which point the experiment was terminated. Plants had grown for 313 d since germination, either entirely in the greenhouse or split into greenhouse (91 d), outdoors (132 d), and then greenhouse again (90 d).

Statistical Analyses. All statistical analyses were carried out with SAS® software version 9.3 (SAS 2011). We first evaluated whether or not the plants bolted, if the plants did bolt how long it took, and whether bolting depended on treatment or plant size. Because all vernalized plants bolted, there was no variation within that treatment (the separation problem; Albert and Anderson 1984), making it impossible to run a generalized linear mixed model (PROC GLIMMIX). Therefore, a Fisher's exact test was performed to evaluate the effects of the temperature treatment on the proportion of plants that bolted. Next, a mixed linear model (PROC MIXED) was used to evaluate the effect of rosette size before bolting on the time to bolting. Analyses included either the size in the fall or one of several different spring size measurements. Fall rosette size, measured as total number of leaves, produced the model with the lowest Akaike information criterion value and is presented in our results. Fall plant size, treatment, and a fall plant size by treatment interaction were treated as fixed effects. Population, population by treatment interaction, and maternal plant nested within population were considered random effects. The significance of random effects was tested using likelihood ratio tests. We obtained -2 residual log likelihoods from running the model with and without the random effects, and the difference between those values provided a test statistic distributed as χ^2 with one degree of freedom (Littell et al. 1996). We evaluated size at bolting, and we compared growth rates for vernalized and nonvernalized plants using models that included treatment as a fixed effect and population, population by treatment interaction, and maternal plant nested within population as random effects. For the nonvernalized plants, we also evaluated whether size in the fall or spring predicted whether or not plants bolted using a generalized linear mixed model with a binary distribution and a logit link. This comparison could not be done for vernalized plants, because all vernalized plants bolted (see Results and Discussion). Data were log transformed to meet the assumptions of ANOVA.

Results and Discussion

Vernalization strongly influenced the ability of plants to bolt and flower ($\chi^2_1 = 160.43$, $P < 0.0001$). In the 90-day period between the end of the vernalization treatment and the end of the experiment, 100% of vernalized plants bolted and flowered, whereas only 26% of the 140 nonvernalized plants ($n = 36$) bolted (Figure 1). The time to bolting varied strongly by treatment (Table 2; Figures 1 and 2). On average, vernalized plants bolted 14 d and flowered 27 d after the cold treatment ended (105 and 118 d of active growth, by which we mean total time since germination, excluding the vernalization treatment when the plants were largely dormant). All vernalized plants bolted within 26 d and flowered within 37 d after treatment (117 and 128 d of active growth). In contrast, for the nonvernalized plants that bolted, the average number of days to bolting was 278 d since germination (173 d more of active growth than the vernalized plants), and for those that flowered, the average number of days to flowering was 295 d since germination (177 d more of active growth than the vernalized plants).

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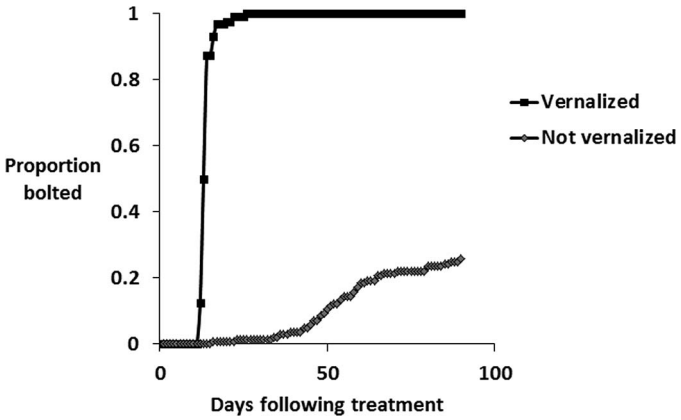

Figure 1. Cumulative proportion of plants from the two temperature treatments that bolted in the 90 d after the end of the vernalization treatment. One hundred percent of vernalized plants bolted within 26 d from the end of the cold treatment. Twenty-six percent of nonvernalized plants bolted within 90 d after treatment, or after 313 d of active growth.

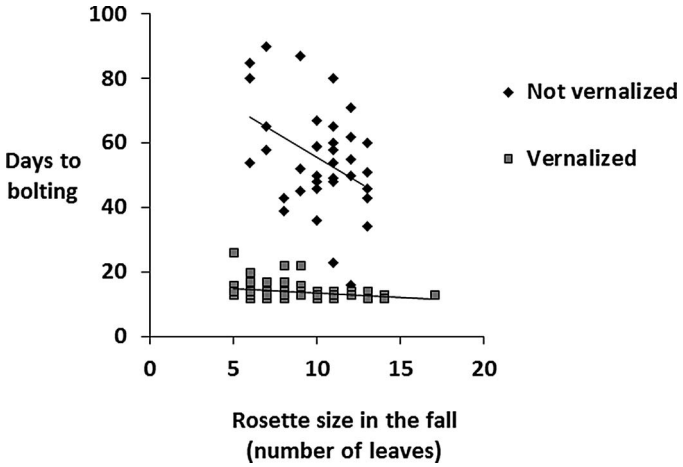

Figure 2. Number of days to bolting after the end of the vernalization treatment for plants in the two temperature treatments, based on fall rosette size. Nonvernalized plants that had more leaves in the fall bolted earlier in the spring than nonvernalized plants that had fewer leaves in the fall. Nonvernalized plants took longer to bolt than vernalized plants, in general. Number of leaves in fall*treatment is significant at P = 0.0246 (Table 2).

The size of the rosette in the fall, before treatments were imposed, influenced time to bolting in the spring, and that influence differed in strength depending upon treatment (Table 2). Across both treatments, rosettes with more leaves in the fall bolted more quickly in the spring, but that pattern was stronger for nonvernalized plants (Figure 2). Even very small vernalized plants could bolt quickly, whereas only very large nonvernalized plants bolted in less than 26 d since plants were recombined into the greenhouse, or at 250 d of active growth (at which time all vernalized plants had bolted). Despite the important role of size in time to bolting of nonvernalized plants, size in the fall and spring did not predict whether or not those plants bolted (fall $F_{1,111} = 0.55$, P = 0.4601; spring first measure $F_{1,111} = 0.05$, P = 0.8259; spring second measure $F_{1,111} = 0.43$, P = 0.5130).

Table 2. ANOVA results from the greenhouse experiment with black henbane, evaluating the influence of fall plant size, treatment, and their interaction on the time to bolting in the spring. The interaction between rosette size (measured as number of leaves) in the fall and treatment was significant. Fall rosette size of vernalized plants did not have a strong effect on time to bolting in the spring. However, nonvernalized plants that were larger in the fall bolted earlier than smaller nonvernalized plants.

Fixed effects	*df*[a]	*F*	P
No. of leaves in fall	1, 136	22.96	<0.0001
Treatment	1, 3	123.43	0.0016
No. of leaves in fall*treatment	1, 136	5.16	0.0246
Random effects	***df***[a]	**Likelihood ratio**	**P**
Population	1	0.5	0.2398
Population*treatment	1	0.1	0.3759
Plant(population)	1	0	0.5

[a] Abbreviation: df, degrees of freedom.

Models that included the other measurements of rosette size (results not shown) were consistent with the findings for fall rosette size data (Table 2), with larger plants always bolting earlier. Plant size at time of bolting differed significantly between treatments ($F_{1,3} = 117.88$, P = 0.0017). On average, vernalized rosettes bolted at a smaller size than their nonvernalized siblings (Figure 3). At bolting, nonvernalized rosettes reached an average size of 41 leaves, whereas vernalized rosettes bolted with an average of 28 leaves. Rosette size for both treatments is measured as the total number of leaves at the time of bolting (i.e., fall rosette size was not added to the vernalized plants, which lost aboveground biomass during the cold treatment).

Growth rates of vernalized and nonvernalized plants were calculated for the week between February 12 and February 19, the second week after the end of the treatment. After vernalization, plants added leaves 1.7 times faster than the plants that had not been vernalized, although that difference was not significant ($F_{1,3} = 3.43$, P = 0.1611).

The fact that no plants flowered in the 90 d before the start of vernalization and the rapid flowering of vernalized plants after their return to the greenhouse, clearly support the hypothesis that our study populations comprise biennial henbane. However, the substantial percentage of plants that did eventually flower, even without vernaliza-

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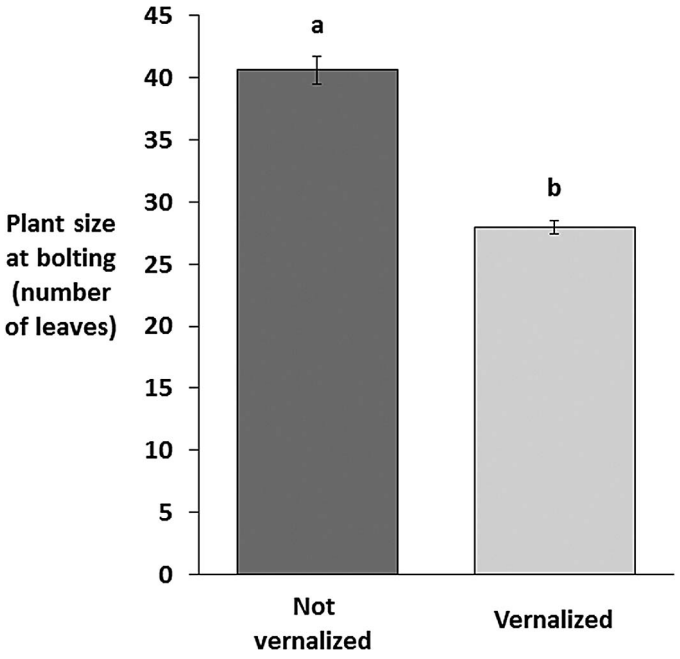

Figure 3. Mean number of leaves at bolting as a function of treatment. Data are means ±1 standard error bars, with different lowercase letters indicating that the means differ significantly at $P < 0.001$.

tion, requires explanation. We propose and discuss three alternative hypotheses to explain the flowering of nonvernalized plants.

1. Late-flowering annuals, like those found by Schläppi (2011), are present in the introduced populations. In his study, 100% of late-flowering annuals bolted within 60 d without vernalization, whereas in ours, only 26% bolted, and that after a much longer 278 d. While an important hypothesis to consider, the long time to flower does not follow the life cycle of a late-flowering annual; thus, this hypothesis is not supported by the current data.
2. Genetic variation weakening the requirement for vernalization may exist in the western United States that did not exist within Schläppi's samples. Schläppi (2011) was able to produce late-flowering winter annual black henbane plants from crossing biennial and annual accessions. It might be that hybridization between biennial and annual forms and subsequent backcrosses to biennials could lead to populations with weaker requirements for vernalization. No data are available currently to address this hypothesis.
3. Aspects of our experimental setup weakened the vernalization requirement. The two main differences other than cold between our vernalized and nonvernalized treatments are differences in the soil moisture content and in the light regime. As previously mentioned, vernalized plants received water via snowmelt during the treatment. Nonvernalized plants were actively growing in the greenhouse and were watered as required, which was typically three times per week. Therefore, nonvernalized plants in the greenhouse had higher soil moisture content than the vernalized plants for 19 wk. There is no evidence from the literature that increased water availability can substitute for vernalization. The light regime in the greenhouse may have played a role, because photoinduction is a known replacement for vernalization in other systems (e.g., *Arabidopsis*; Bagnall 1993). Nonvernalized plants in our experiment received more light than the vernalized plants during the treatment period, with the greenhouse lights set to long days and short nights, the ideal photoperiod for flowering of black henbane (Downs and Thomas 1982; Lang 1986; Parker et al. 1950; Schläppi 2011). Thus, the long photoperiod and higher light intensity in the greenhouse relative to undermulch outside could have caused some nonvernalized biennial plants to flower. This hypothesis seems plausible, given the known mechanism from other systems, but additional data would help distinguish among the three.

Our interpretation that introduced populations in Colorado, Wyoming, and Montana are dominated by biennial plants is supported by field data from more than 25 populations across those states. Germination took place in June, and marked plants did not flower until the following summer (C. Fettig, unpublished data). An additional line of evidence comes from the current distribution of black henbane in North America; the U.S. Department of Agriculture PLANTS Database (USDA 2014 reports this species to be found exclusively in areas that experience at least 10 wk of cold winter temperatures, suggesting that vernalization is required in natural populations.

Our results indicate that the plants from our sampled, introduced populations are strongly biennial. We believe this bodes well for land managers, because it likely limits the areas that henbane could invade. Although our experiment demonstrated that vernalization is not absolutely required for bolting and flowering, it seems unlikely that seeds transported to climates without a winter cold period would establish successfully. In the greenhouse conditions, plants received sufficient resources (water, nutrients, light). However, in natural areas throughout much of the United States this would rarely be the case, with water being limiting at some period over the course of nearly 300 d in the West and Southwest and other resources being limiting due to competition in the Southeast. Indeed, henbane is found primarily in highly disturbed, open habitats (C. Fettig, unpublished data) and is a poor competitor (LaFantasie and Enloe 2011). Therefore, it may be that the current distribution of black henbane in North America, which reflects the biennial life

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cycle we document here, represents a reasonably stable range, as long as an annual form is not introduced.

Despite current efforts to prevent introductions of nonnative species, introductions continue to occur (Cohen and Carlton 1998; Levine and D'Antonio 2003; Pysek et al. 2003). USDA-APHIS requires a "Permit to Import Plants and Plant Products" for any work with black henbane, along with a declaration that the seed is for research purposes only (USDA-APHIS 2014). However, black henbane is desired as an ornamental planting, as well as for medicinal uses, and as such is cultivated in gardens still today. Given this, henbane seeds are readily available for purchase without a permit through Internet vendors worldwide, despite the USDA-APHIS regulations. Furthermore, interstate movement of the plant is not regulated at the federal level. Given the commercial sale of seeds, eventual introduction of the annual form may be inevitable. However, Mitich (1992) reports that herbalists prefer the more productive and alkaloid-rich biennial form of the plant, providing some hope that the annual is not commonly planted. If introduction of the annual forms can be prevented, then the habitable range of black henbane in North America may be restricted to northern climates.

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