Seed vernalization and gibberellic acid interact to affect life cycle type in facultative winter annual Canadian horseweed (*Erigeron canadensis*)

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Abstract

- *Background and Aims:* Plants display enormous variation in the phenological traits that make up their life cycle both within and between populations. Facultative winter annual species are particularly interesting because they can adopt either a fall-emerging/spring-flowering or spring-emerging/summer-flowering life cycle at the population level via evolution or at the individual level via within-generation and transgenerational plasticity. We hypothesized that the growth hormone gibberellic acid (GA) may be a mechanism by which these plants adjust their life cycle in response to cold temperatures.
- *Methods:* We conducted growth chamber and greenhouse experiments using the facultative winter annual agricultural weed *Erigeron canadensis* (horseweed) to investigate the interactive effects of genetic variation; transgenerational responses to parent plant life cycle; and plastic responses to temperature, light, and GA treatments.
- *Key Results:* We found that contrary to a prior report, exposing imbibed seeds to 3-4 weeks of cold (i.e., seed vernalization) does not always result in summer annual type growth, with considerable variation found among field-collected seeds from 10 populations. Further, we found that seed vernalization and exogenous application of GA both tended to increase summer annual characteristics, interacting in ways that were largely consistent with the hypothesis that GA is a mechanism for cold-induced life cycle differentiation. Light treatment did not significantly affect life cycle traits, while parent life cycle type had marginal effects on offspring life cycle type. Finally, genetic variation among and within sites explained far less of the variation in life cycle traits than the plastic responses to seed vernalization and GA treatments.
- *Conclusions:* Our study proposes that the seasonality of this harmful agricultural weed is influenced by a GA-mediated response to vernalization of seeds during winter, yet highlights the need for further study, given the variability in this response.

Key Words: weed ecology, phylogeny, life cycles, plasticity, genetic variation, Erigeron canadensis, Conyza canadensis, horseweed, marestail, seed vernalization, gibberellic acid (GA), winter annual

Introduction

Phenology is the timing of recurring events in organismal life cycles. Phenological traits in plants include time of emergence, flowering, and senescence. The practical applications of studying plant phenology have long been recognized by agricultural scientists (Huberman 1941), and a growing number of studies in recent decades have focused on documenting phenological shifts in response to anthropogenic climate change (Forrest and Miller-Rushing 2010). Such shifts often serve as crucial mechanisms by which plants become or remain well-adapted to their environments. Plants may plastically adjust their phenology in response to environmental cues received during their own (within-generation plasticity) or their parent's (transgenerational plasticity) lifetime (Auge et al. 2017). In addition, plant populations may evolve in response to selection on phenological traits (e.g., Hall and Willis 2006).

While incremental changes in plant phenology have been widely studied, categorical changes in life cycle type have received less attention. Plants are typically classified as annuals if they complete their life cycle in one year, biennials if they complete their life cycle in two years, or perennials if they complete their life cycle in more than two years. Annual plants can be further subdivided into summer annuals, which emerge in spring and reproduce by fall; and winter annuals, which emerge in fall, overwinter as a rosette, and reproduce from spring to summer. Facultative winter annuals can adopt either a winter or summer annual life cycle in response to different environmental conditions at the level of the population via evolution (Meyer et al. 2004, Bloomer and Dean 2017, Charbonneau et al. 2018) and at the level of the individual via plasticity (Sans and Masales 1993, Lu et al. 2014). Both mechanisms often co-occur (Best and Mc Intyre 1976, Landers 1995, Garrison 2024). Furthermore, nongenetic transgenerational plasticity (i.e., parental effects) may also play a role in determining offspring life cycle type (Mennan and Ngouajio 2006, Lu et al. 2016, Kanomanyanga et al. 2025). The effects of all of these factors on life cycle differentiation are rarely studied together, making it difficult to compare their importance or look for interactions between genes and environment.

A change to the biosynthetic regulation of the plant growth hormone gibberellic acid (GA) is a promising proximal mechanism by which environmental or genetic variation influences life cycle differentiation. This hormone has been found to play a key role in germination, stem elongation, and flowering initiation (reviewed in Gupta and Chakrabarty 2013; and in Shah et al. 2023), which are all important parts of life cycle differentiation. Exogenous GA application has been found to override normal photoperiod or temperature requirements for flowering in at least 42 species of rosette-forming summer annual, winter annual, biennial, and perennial plants (reviewed in Lang and Reinhard 1961; and in Zeevaart 1983). One study showed that the exogenous application of GA accelerated the flowering of winter annual, but not summer annual ecotypes of *Raphanus raphanistrum*, suggesting GA upregulation as a mechanism of evolved rapid summer annual flowering (Garrison 2022). Further, *Chrysanthemum morifolium* summer-flowering mutants were found to have higher endogenous levels of GA than normal fall-flowering plants (Deng et al. 2017).

The present study uses the facultative winter annual *Erigeron canadensis* to investigate the contributions of genetic variation, transgenerational plasticity, within-generation plasticity, and hormonal regulation to life cycle differentiation in this species. Results from a prior study suggest that winter vs. summer annual life cycle differentiation can be explained as a plastic response to seed vernalization, in which imbibed seeds are exposed to cold conditions that simulate winter, similar to seed stratification but used to induce flowering rather than break dormancy (Schramski et al. 2021). The authors subjected seeds sourced from summer annual parents growing in two Michigan agricultural fields at each of two sites to various temperature, photoperiod, intraspecific competition, light level, soil moisture, and vernalization treatments and found that only seed vernalization affected life cycle type, with four weeks at 4ºC sufficient to cause all plants to be classified as summer annuals. Despite this compelling finding, it is limited by having few populations, few individuals per population, and only one parental environment, meaning that possible genetic and transgenerationally plastic contributions to the response remain unclear.

We first tested whether cold-vernalizing seeds would produce summer annual plants across a broader sample of populations from Michigan, grown in a common greenhouse environment (Fig. 1A). We then conducted a growth chamber experiment in which we crossed the seed source factors of population, line, and parent type (field summer annual, greenhouse summer annual, or greenhouse winter annual) with seed vernalization and gibberellic acid treatments (Fig. 1B). As in Schramski et al. (2021), we grew plants until the juvenile stage and categorized them by appearance into life cycle types. Given the relatively continuous range of variation we observed, we complemented the categorical data with quantitative measures of leaf roundness and plant height growth rate, since rosette-forming winter annuals make rounder leaves and remain close to the soil surface prior to bolting. In our follow-up greenhouse experiment, we grew a smaller sample of plants but followed them until senescence, allowing us to measure the critical life cycle trait of flowering time (Fig. 1C). In this experiment, we tested the crossed effects of seed vernalization, GA, and light level. We also included seeds collected from one of the same sites used in Schramski et al. (2021) to rule out the possibility of divergent results between our experiments arising from population-specific responses.

We made the following hypotheses and predictions: 1) We hypothesized that life cycle type differentiation in horseweed is primarily plastic based on Schramski et al.'s consistent results among their two collection sites, and thus we expected to find little variation explained among or within populations. 2) We hypothesized that plastic growth type differentiation could be explained by a combination of a) parent life cycle type, b) offspring seed temperature, and c) offspring light environment. Specifically, we predicted that summer annual characteristics would be greatest in offspring of winter annual parents, seeds exposed to winter-mimicking seed conditions, and plants grown in sunny conditions given that summer annual plants germinate in the spring with little competitor biomass present after winter. 3) We hypothesized that the effects of seed vernalization are at least partially mediated by upregulation of GA. Based on this, we first predicted that plants treated with GA would be more likely to show summer annual characteristics. We then predicted that GA would have little effect on seed-vernalized plants already upregulating GA, and seed vernalization would have little effect on plants already receiving increased GA.

Overall, our study aims to provide a more complete picture of the factors that influence variation in horseweed life cycle type, serving as a useful case study for future studies of life cycle types in other species and providing insight for managers of this harmful agricultural weed.

Materials and Methods:

Study System

Erigeron canadensis L. (syn. *Conyza canadensis*, Canadian horseweed, or marestail; hereafter horseweed) is a weedy annual plant in the Asteraceae family that is native to Central and North America (Weaver 2001). It now commonly infests agricultural fields and disturbed habitats in temperate zones across the world. Horseweed forms wind-dispersed, primarily self-fertilized seeds (estimated at 96%, Smisek 1995). It is considered a facultative winter annual because it can adopt a winter annual or summer annual life cycle. While there seem to be peaks of emergence in the fall and spring, the species has wide emergence and flowering windows (Main et al. 2006, Tozzi and Van Acker 2014). Plants classified as winter annuals generally form a low-to-the-ground rosette for overwintering, whereas those classified as summer annuals skip the rosette stage by immediately growing upright. However, there is continuous variation in the degree to which plants form a rosette and the life cycle types are not morphologically distinguishable at flowering, since any rosette leaves have senesced by this point. Highly contrasting proportional spring vs. fall emergence has been reported from nearby sites (Main et al. 2006), and there does not appear to be a clear latitudinal pattern in life cycle proportions (e.g. 4-24% summer annuals at 36ºN, Main et al. 2006; 92-100% at 39ºN, Davis and Johnson 2008; 38-32% at 45ºN, Buhler and Owen 1997). However, these studies demonstrated that the two life cycle types usually co-occur in the same field. There are also observational reports that the summer annual life cycle is becoming more common in Michigan (Schramski et.al. 2021).

2021 field collections and 2022 greenhouse common garden

In the fall of 2021, seeds were collected from 30 individuals from each of 10 sites (except for 1 site with just 23), spread across Michigan's lower peninsula (see Fig 1A and Table S1 for site details). These 293 field parents were presumed summer annuals given their seed production in fall. The self-fertilized seeds produced by a given parent plant are likely highly homozygous so we refer to them as lines. Seeds were stored in envelopes with desiccant at 5°C until used. In May 2022, approximately 50 seeds per line were sown in 200-cell plug trays and vernalized in a growth chamber set to 4ºC and 8-hour daylength for three weeks. Trays were then moved to a greenhouse at the Kellogg Biological Station (Hickory Corners, MI), thinned to up to five seedlings per line, and transplanted at the first true leaf stage into 16.5-cm-diameter pots filled with SureMix potting soil (Michigan Grower Products, Inc.). Pots were thinned to one plant when leaves started to substantially overlap. Plants that had not bolted by the end of August (about 11 weeks since movement to greenhouse) were vernalized for 35 days in a growth chamber in the same conditions as those used for seed vernalization. A fungicide soak was applied to the vernalized plants to prevent the spread of fungal root rot. Plants were assigned to one of three categorical growth types based on visual assessment by the same observer about 1 week after emergence and updated about 9 weeks after emergence. "Rosette" types had a basal circular arrangement of dark green, round leaves; "Upright" types had lighter and more elongated leaves arranged along a visible stem (i.e., caulescent); "Intermediate" types fell somewhere between the Rosette and Upright types in their characteristics. Of the 293 sown lines, 228 produced a plant that survived to flower.

2023 Growth Chamber Experiment

In this experiment, we tested the effects of seed vernalization and gibberellic acid treatments, along with the seed source factors of population, line, and parent type on juvenile plant life cycle type traits (Fig. 1B). Seeds were sourced from six lines from each of a subset of four of the sites (DLF, KNC, NCF, and PCC; Table S1). Because we did not have seeds from the same line exhibiting different life cycle types in the same generation, we included offspring of three parent plant groups: 2021 field summer annual, 2022 greenhouse summer annual, and 2022 greenhouse winter annual (those requiring rosette vernalization to flower). Comparing the latter two groups tests for transgenerational parental effects of life cycle type while controlling for other possible transgenerational parental effects, but not for effects of genetic variation due to line identity. Comparing the first and last group tests for transgenerational parental effects of life cycle type while controlling for genetic variation, but not for possible transgenerational effects from the field vs. greenhouse environment. Eighteen replicate seeds from each of the four sites, three families, and three parent source types were randomly assigned to one of two vernalization treatments and one of three GA treatments (detailed below), conducted in two trials, initiated 15 days apart (4 sites x 3 parent types x 3 lines x 2 vernalization treatments x 3 GA treatments x 3 individuals x 2 trials = 1296 total). A total of 846 seeds emerged and survived until data collection (65%).

We tested the effects of GA applied as a seed soak, a cotyledon spray, neither, or both. Both trials included 0, 1 and 2 applications of GA, but due to limited space, we could not include all four combinations of seed soak and leaf spray in both trials. In Trial 1, the single GA application was the seed soak, while in Trial 2 it was the leaf spray. Our results focus on the comparison between plants that received no GA ("**Water**") with those that received both the GA seed soak and GA leaf spray ("**GA**"), which was replicated across trials. Seeds were soaked in either a GA solution (1 g/L, Purdom and Glover 2017) or in distilled water for 24 hours at room temperature. At the point of cotyledon emergence, seedling leaves were sprayed with approximately 0.25 mL of either a GA solution (0.035 g/L = 10^{-4} M, Khan et al. 2006) or distilled water.

After completing their soaking treatments, seeds were sown onto the surface of 200-cell plug trays filled with moistened SureMix potting soil, with one tray per treatment (3 GA treatments x 2 vernalization treatments x 2 Trials = 12 total trays) and randomized positions within trays. **Vernalized** seeds were placed in a simulated winter growth chamber set to 4ºC and 9-hour daylength. **Unvernalized** seeds were placed in a simulated summer chamber set to 24ºC and 15-hour daylength. Although in the field we expect seeds to primarily emerge in fall or spring, the unvernalized seeds sown in simulated summer conditions readily emerged (Fig. S1). Surprisingly, some seeds in simulated winter conditions also emerged in the cold (Fig. S1). Accordingly, we had to end the planned 4-week vernalization period early (after about 40% of seeds had emerged, 3 weeks in Trial 1 and 1 week in Trial 2) to prevent cold-related mortality. After their vernalization treatments, both growth chambers were set to 17ºC and 12-hour daylength to simulate spring/fall equinox conditions (based on local average max temperatures and daylengths in mid-fall and mid-spring).

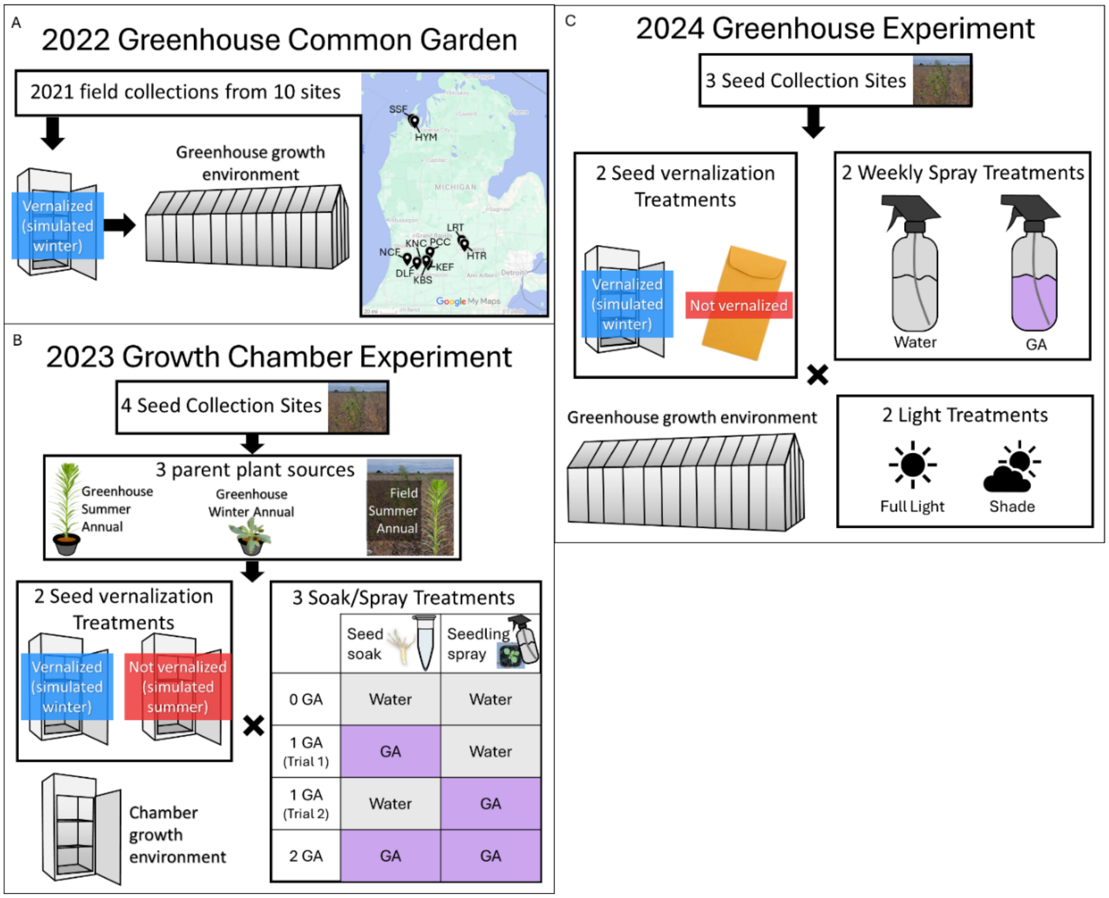
Pots were censused every day for newly emerging seedlings (final seedling emerged on day 33 in Trial 1 and day 18 in Trial 2). Seedlings were defined as having emerged once a stem and both cotyledons were visible. After 3 – 5 weeks of growth, each individual was assigned a categorical growth type (as in 2022 greenhouse common garden), and measurements were taken of plant height, plant diameter, and leaf roundness on the same day by vernalization treatment. Vernalized plants were measured 16 days after unvernalized plants, enabling measurements to be taken after the same mean number of days since emergence in Trial 1, but 11 days apart in Trial 2 (staggering measurement points could not be done due to logistical constraints). Height was measured with a ruler as the vertical distance between the soil and the last node (meaning that height was close to 0 for a rosette structure). Overhead photographs and manual tracing in ImageJ were used to measure maximum plant diameter and leaf roundness (4 x area/[pi x major axis^2], ranging from 0 to 1, with 1 being a perfect circle) on one leaf/plant for all plants with at least one clearly visible entire leaf (N = 785). Height growth rate was calculated as height in mm/days since emergence (results from alternative calculation as height in mm/diameter in mm provided in supplemental). Although some plants were measured on different days since emergence, our analyses examine height growth rate rather than absolute height, and leaf shape rather than size. Both measures are quantitative indicators of life cycle type, since plants forming a rosette stay close to the ground prior to bolting and form rounder leaves.

2024 Greenhouse Experiment

In this experiment, we tested the effects of seed vernalization, gibberellic acid, and light treatments, along with the seed source factors of population and line on life cycle traits in plants grown to maturity (Fig. 1C). Seeds were sourced from two to three lines from each of two sites from the 2022 greenhouse generation (HTR and LRT) plus five lines from fall 2023 collections from one of the same fields used in Schramski et al. (2021, MSU Agronomy Farm, Lansing, MI) using the coordinates they provided (Table S1). For each selfed line, pots (7-cm-diameter) were randomly assigned to one of eight treatment combinations, detailed below, with 5 replicate seeds per pot. Pots were filled with SureMix potting soil. Of the 400 seeds planted, 26% emerged and of those that emerged, 46% survived to flower (substantial mortality resulted from fungal root rot).

For **Vernalized** pots, soil was soaked and then the 5 seeds were spread on the surface of the soil. The pots were placed in randomized positions in a growth chamber set to 4°C and 8-hour daylength. Pots were checked weekly for any emerging seedlings and to ensure moist soil conditions. After four weeks, pots were removed from the growth chamber and placed in a greenhouse (Kellogg Biological Station, Hickory Corners, MI). On the same day, **Unvernalized** seeds were planted in pots in the same manner as the vernalized pots and placed in the greenhouse. Note that in this experiment, the vernalization control was untreated seeds because we wanted plants in both treatments to germinate and develop at the same time. Pots were placed on the same greenhouse bench in randomized positions within one of two light treatments: **Shade** pots were placed under a shade tent (shade cloth over PVC pipes, mimicking fall emergence among abundant vegetation), **Full Light** plants were placed in the open (mimicking spring emergence among sparse vegetation). Shaded plants received about 76% less photosynthetically active radiation (based on 20 measurements per treatment with an Accupar LP-80 ceptometer). Plant leaves were sprayed weekly with either **Water** (distilled) or **GA** solution (0.035 g GA_3/L distilled water). All pots were kept well-watered and maintained until all plants had either died or flowered.

Pots were censused every 4 days for newly emerging seedlings for the first 16 days, and every 7 days for the next 7 weeks, at which point no further emergence occurred. Plants were also censused weekly for signs of flowering, and plants were marked as having flowered if they contained at least one fully expanded flower. Plant height was measured as the length of the stem from the base of the soil to the last node on days 21, 49, and 64 since planting, along with at the first census in which they flowered. We defined height growth rate as day 64 plant height in mm /days since emergence. Nine weeks after planting, plants were assigned a categorical growth type and leaf roundness was estimated from photos of the largest leaf from one plant per line.



	Seed soak	Seedling spray
0 GA	Water	Water
1 GA (Trial 1)	GA	Water
1 GA (Trial 2)	Water	GA
2 GA	GA	GA

Figure 1. Overview of experimental designs for 2022 Greenhouse Common Garden (A), 2023 Growth Chamber Experiment (B) and 2024 Greenhouse Experiment (C).

Statistical Analyses

All statistical analyses were done in RStudio running R v. 4.4.2 (R Core Team 2024). The package "emmeans" (Lenth 2024) was used to test significance of model terms (Type III Analyses of Deviance), conduct post-hoc analyses on treatment combination means, and generate estimated marginal means. All plots were made using the package "ggplot2" (Wickham 2016). The package "MuMIn" (Bartoń 2025) was used to calculate marginal and conditional R^2 values for our linear mixed effects models, where marginal is the variance explained by the fixed effects and conditional is the variance explained by both fixed and random effects (Nakagawa and Schielzeth 2012). The difference between conditional and marginal was used to estimate the additional variance in the response explained by adding the random effect of line.

For the 2023 growth chamber experiment, we used linear mixed effects models with the response variables of height growth rate (log-transformed to improve normality of residuals) and leaf roundness; the fixed effects of GA treatment (GA vs. Water), seed vernalization treatment (vernalized vs. unvernalized), trial (1 vs. 2), and all two- and three-way interactions plus parent plant type (greenhouse winter annual vs. greenhouse summer annual vs. field summer annual), seed source site, and the temperature at which a plant emerged (4 vs. 17 vs 24 °C); and the random effect of selfed line. We ran similar models except that we replaced GA treatment with number of GA applications to look for different effects of the GA seed soak vs. leaf spray.

We used a similar approach to model height growth rate, leaf roundness, and days to first flower in the 2024 greenhouse experiment, except that a log transformation was not needed to get normally-distributed residuals, greenhouse light treatment (full light vs. shade) replaced trial, and parent type and emergence temperature were removed. Additionally, for the leaf roundness model we removed the three-way interaction since the model did not converge when it was included, given the reduced sample size from subsampling leaf measurements.

For our categorical growth type variable, given that the frequency of Intermediate was small and unbalanced, we analyzed two binary growth type variables, either grouping Intermediate with Upright or Rosette. Results for the two grouping methods were quantitatively similar so we only report the latter grouping. Logistic regressions did not converge due to probabilities of 1 or 0 in some groups, so instead we ran pairwise two-sample Fisher's exact tests separately by the fixed effects in our models listed above. For fixed effects with more than two levels, we adjusted P-values for multiple testing using the Benjamini-Hochberg procedure to control the False Discovery Rate.

Results

Hypothesis 1: Genetic differentiation in life cycle type

In our 2022 greenhouse common garden, the life cycle type of plants sourced from field-collected and vernalized seeds varied significantly among 10 collection sites in Michigan, explaining 15% of the deviance in the response from a saturated model (Fig. S2A, $P = 4.8E^{-5}$). However, the patterns do not appear to track latitude or average winter temperatures (Table S1; e.g., lack of similarity between nearby DLF and KNC sites). Because we grew field-collected seeds in a common environment, these differences reflect some combination of evolved genetic differentiation and transgenerational parental effects resulting from variation in field environments.

We then grew offspring of these 2022 greenhouse plants alongside offspring of field parents from a subset of four sites in our 2023 growth chamber experiment. In our 2024 greenhouse experiment we grew greenhouse offspring from a subset of two sites, plus offspring of field parents from a field site used by Schramski et al (2021). In both experiments, source site did not significantly affect any of our measured variables in either experiment (Fig. S2B,C and see Fig. 3 boxes for Site effect P-values). This lack of a significant site effect held when including only offspring of greenhouse parents ($P > 0.23$).

In both experiments, we also included multiple genetic lines from each site, expected to be highly homozygous in this selfing species. Line explained a significant amount of variation in height growth rate and leaf roundness only in the growth chamber experiment. Even so, line explained only 2% and 4% of the variance, compared with 37% and 27% explained by GA and vernalization treatments for height growth rate and leaf roundness, respectively. The lack of variation in life cycle type explained by variation between and within populations is consistent with the hypothesis that life cycle is primarily determined via plasticity.

Hypothesis 2a: Transgenerationally plastic response to parent life cycle type

In the 2023 growth chamber experiment we included seeds generated by parents exhibiting the summer annual growth type in the greenhouse, winter annual growth type in the greenhouse, or summer annual growth type in the field. We predicted that offspring of summer annual parents would be more likely to exhibit a winter annual life cycle than offspring of winter annual parents, enabling an alternation of life cycles. Parent type had a small but significant effect on height growth rate, where offspring of greenhouse-grown summer annuals had slightly greater height growth rate compared with the other two types. Therefore, there was only a significant difference for one of two parent life cycle comparisons (in which other environmental variation but not genetic line was controlled) and the effect was opposite our prediction of parent-offspring mismatch. There was also a small but significant effect of parent type on leaf roundness, which was driven by offspring of field-grown summer annuals having slightly rounder leaves than offspring of greenhouse-grown winter annuals (with offspring of greenhouse-grown summer annuals in between the other two). The pattern was therefore in the predicted direction of parent-offspring mismatch for both comparisons but only significant for one of them. Categorical assignment to the upright summer annual type was similar among the three parent types ($P = 0.78, 0.90, 0.98$ for the three comparisons).

Hypothesis 2b: Plastic responses to seed vernalization

Contrary to our prediction that all cold-vernalized seeds would develop as summer annuals, when seeds were vernalized for three weeks and grown in a common greenhouse environment, an average of 65% of plants formed an overwintering rosette structure indicative of a winter annual life cycle. Similarly, 66% of plants only flowered after receiving additional cold through rosette vernalization, after failing to bolt for 11 weeks (87% of these were Rosette types; Fig. S2A).

In the 2022 and 2023 experiments, the overall effects of seed vernalization were also variable, but tended to increase summer annual type characteristics, in line with our prediction. Specifically, seed vernalization increased summer annual characteristics for all measures in the greenhouse experiment (though not significantly in the case of leaf roundness; Fig. 2B, Fig. 3C-E) and for leaf roundness in the growth chamber experiment (Fig. 3B), but not for height growth rate in the growth chamber experiment (Fig. 3A).

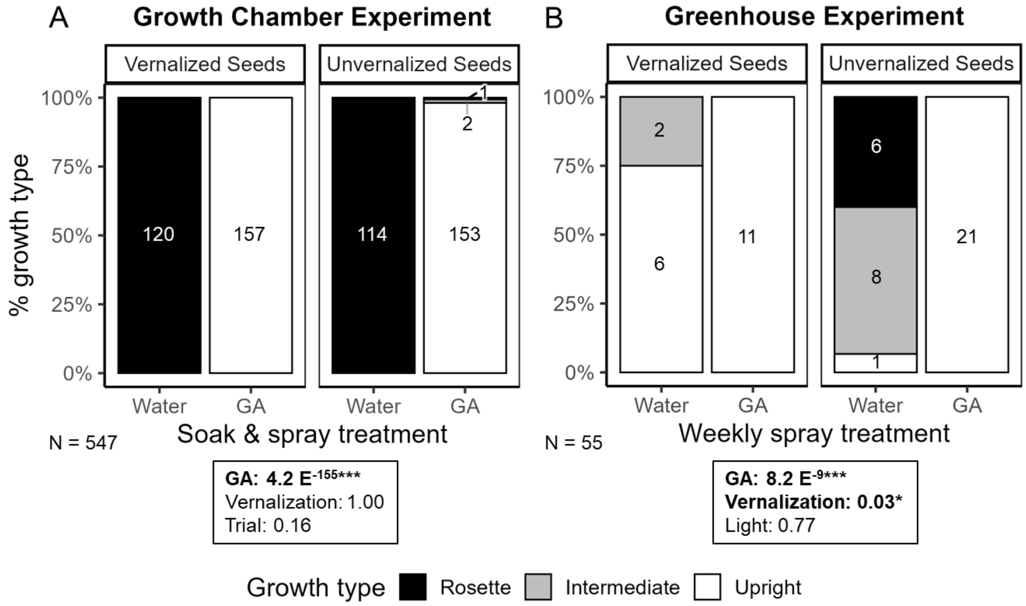


Figure 2. Qualitative measure of life cycle growth type in 2023 Growth Chamber (A) and 2024 Greenhouse (B) Experiments. Colored bars show the proportion of plants categorically assigned to Rosette (black), Intermediate (grey), or Upright (white) growth, with counts shown within bars. Panels separate seed vernalization treatments. Numbers in bars are counts. The boxes show Fisher's Exact Test *P*-values for the indicated groups.

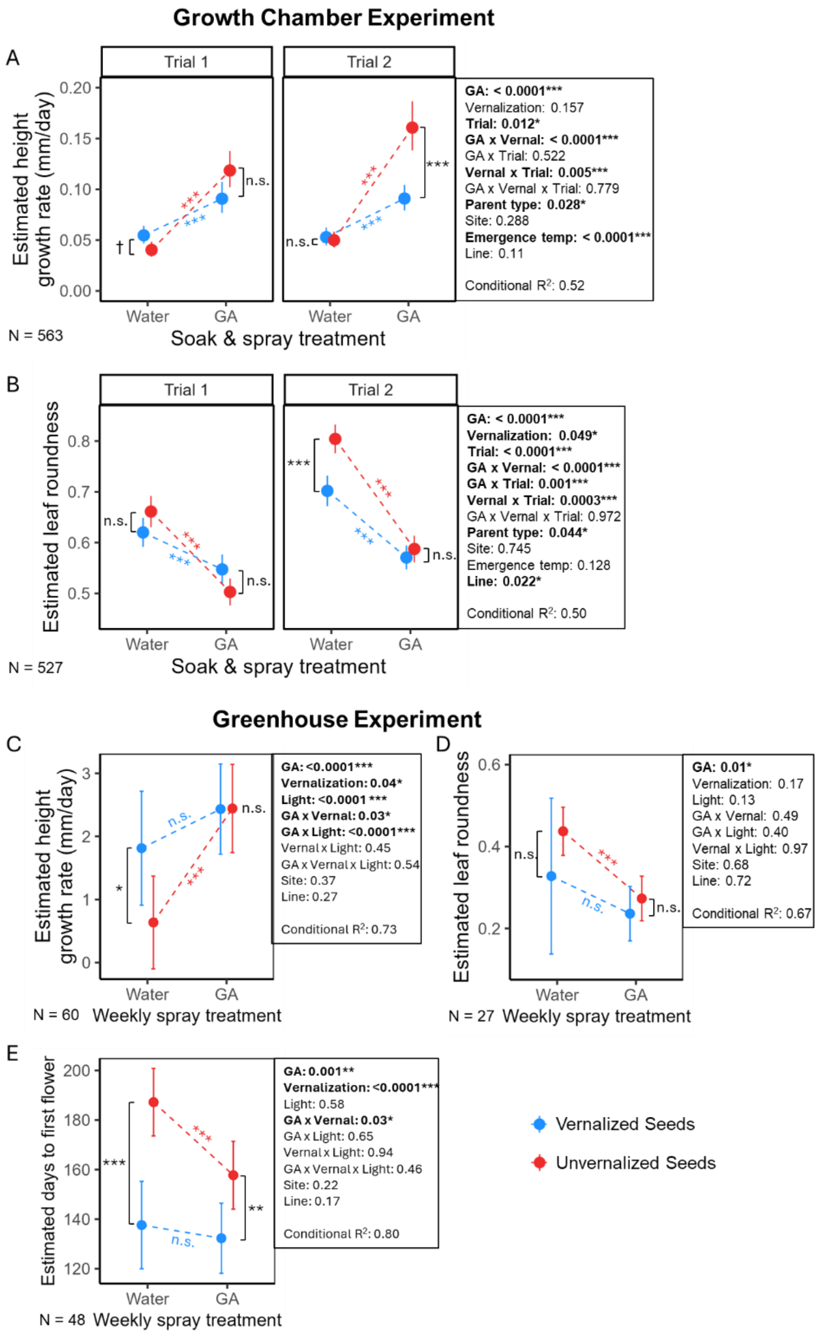


Figure 3. Quantitative measures of life cycle type in 2023 Growth Chamber (A and B) and 2024 Greenhouse (C to E) Experiments: height growth rate (A and C), leaf roundness (B and D), and flowering time (E). Colors separate seed vernalization treatments and the slope of dotted lines is the effect of adding GA within vernalization treatment. Panels separate trials in A and B. Points are estimated marginal means after accounting for the other effects in the model and error bars are 95% confidence intervals. The box gives the *P*-values for all model effects from a Type III Analysis of Deviance for fixed effects and likelihood ratio test for the random effect of Line. Asterisks on lines indicate significance of GA effect within vernalization groups, while asterisks between points indicate significance of vernalization effect within soak & spray treatment groups from Tukey post-hoc tests. $^{\text{n.s.}}P > 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Hypothesis 2c: Plastic response to offspring light environment

In the 2024 greenhouse experiment, we tested our prediction that shaded plants would be more likely to develop winter annual characteristics, particularly if seeds were not vernalized; that is, mimicking late summer or fall germination of seeds produced that year. However, light treatment did not significantly affect growth type assignment ($P = 0.77$), leaf roundness ($P = 0.13$), or days to first flower ($P = 0.58$), and did not significantly interact with vernalization treatment for any measure ($P > 0.45$). While shaded plants had lower overall height growth rate (-1.7 mm/day) and were less affected by GA spray, given that light treatment did not affect any other measure of life cycle type, we interpret this result as simply reflecting the stunted growth expected from light-limited plants, rather than the limited elongation of rosette-forming plants.

Hypothesis 3: Seed vernalization response mediated by upregulation of GA

In line with our predictions, the application of GA strongly and significantly increased all of our measures of summer annual type growth across both experiments. Specifically, plants exposed to exogenous GA were more likely to be assigned the Upright growth type (Fig. 2), extended their height at a faster rate (Fig. 3A,C), had more elongated leaves (Fig. 3B,D), and flowered sooner (Fig. 3E). This pattern held in the growth chamber experiment when excluding plants in the vernalization treatment that unexpectedly emerged while still in the cold (Fig. S3) or when quantifying growth rate as plant height per plant diameter rather than per day (Fig. S4). Across our life cycle measures, applying GA only to seeds rather than only to leaves generally had a weaker effect (Fig. S5), but this comparison was not replicated and so should be interpreted with caution.

We also predicted that if GA upregulation is a mechanism by which seed vernalization influences life cycle type, then the two treatments would be at least partially redundant. This would manifest as a statistical interaction between GA and vernalization treatments whereby each factor would have a stronger effect in the absence of the other. We found this predicted pattern for all measures in the greenhouse, but only for leaf roundness in the growth chamber. Specifically, in the greenhouse experiment, seed vernalization in the absence of GA or GA in the absence of seed vernalization caused nearly all plants to be assigned Upright (interaction not statistically testable, Fig. 2B). For leaf roundness in both experiments, along with flowering time and height growth rate in the greenhouse experiment, the vernalization effect was larger in magnitude in the water control group compared with the GA-treated group (compare differences between red and blue points within treatments in Fig. 3B-E; although note that this was not significant for leaf roundness in the greenhouse). Similarly, for these measures, the GA effect was larger in magnitude in the unvernalized group than the vernalized group (compare slopes of red and blue lines in Fig. 3B-E). However, seed vernalization did not affect growth type assignment within either the GA or Water groups in the growth chamber experiment. For height growth rate in the growth chamber experiment, the magnitude of the GA effect was larger in unvernalized seeds as predicted, but the vernalization effect switched from slightly positive in the water-treated group (+0.009 mm/day, $P = 0.07$) to negative in the GA-treated group (-0.05 mm/day, $P < 0.0001$), opposing our prediction. Although the three-way interaction was not significant, we note that this surprising negative effect of vernalization on height growth rate in in the GA-treated group was only apparent in Trial 2, which had the most truncated period of seed vernalization due to widespread germination in the cold (Fig. S1).

Discussion

Life cycle type differentiation is primarily plastic rather than genetic

We found that field-collected seeds from 10 Michigan sites vernalized and grown in a common greenhouse environment varied between and within sites in morphological growth type and requirement for further vernalization of rosettes. However, when we grew offspring of plants from subsets of these sites plus new collections from a site used by Schramski et al. (2021), site differences largely disappeared. For example, sites KNC and DLF were some of the most divergent in the 2022 greenhouse but did not significantly differ in the 2023 growth chamber. Although, we only grew seeds from six of the 10 originally sampled sites, our results suggest that the differences in traits associated with life cycle type found in the first greenhouse generation primarily reflect transgenerational plasticity, rather than evolved genetic differentiation. The low levels of differentiation between presumably highly homozygous families further supports the idea that variation in life cycle type is not strongly predicted by genetic variation. Although Schramski et al. (2021) did not statistically test for differences among their four seed families from each of four fields, the highly consistent responses of all plants to their experimental treatments suggests little genetic variation for life cycle type, consistent with our study. However, genetic differentiation across a broader geographic range in this widely distributed weed remains possible and should be tested in future studies.

Mixed evidence for transgenerational effects of parent life cycle type

In addition to comparing field-collected vs. greenhouse-grown seeds, we also explicitly looked for transgenerational effects of parental life cycle type on offspring life cycle type in our growth chamber experiment. We predicted that differences in the conditions experienced by summer annual versus winter annual parents would reduce parent-offspring resemblance. Alternating parent-offspring life cycles might be adaptive in nature if the parent environment is strongly predictive of the offspring environment (Uller 2008) whereby seeds are shed by winter annual parents in favorable conditions for summer annual emergence, and vice versa. A similar pattern has been found previously in the facultative winter annual *Isatis violascens,* where parent germination season biases seeds towards the dimorphic dormancy type that promotes germination in a different season than the parent (Lu et al. 2016). First, our study confirms that parents of both life cycle types can produce offspring of both life cycle types, whereas this was previously only shown for summer annual type parents (Schramski et al. 2021). We found evidence for a transgenerational effect in the predicted direction for leaf roundness but not height growth rate. Future studies should include field winter annual parents as a comparison group to provide a more complete picture of transgenerational plasticity in life cycle type. Overall, these results suggest that transgenerational plasticity plays some role in life cycle type differentiation, but the specific environmental cues shaping these responses remain unclear.

Some evidence for a plastic response of life cycle type to seed vernalization but no evidence for a plastic response to light level

Given that summer annuals emerge in spring after cold and wet winter conditions, we expected seeds exposed to simulated winter conditions to emerge as summer annuals. Consistent with this expectation, we found that exposing seeds to cold and wet conditions for three to four weeks increased summer annual growth characteristics, but not in all plants. In our greenhouse common garden in which all seeds were vernalized, only about a third of plants developed as summer annuals. Among plants that did not receive GA in our two experiments, all plants developed winter annual characteristics regardless of vernalization treatment in the growth chamber, yet seed vernalization increased the chances of developing summer annual characteristics in our smaller sample of greenhouse plants. These results contrast markedly from those of Schramski et al. (2021), who classified no unvernalized plants as summer annuals, but 88% and 100% of plants vernalized for two weeks and four weeks as summer annuals respectively. That study and all seed vernalization trials in the present study utilized the same temperature (4ºC) and the same or similar photoperiod (8 – 9 hours). These results demonstrate that while seed vernalization plays a role in life cycle determination, its variable effects point to interactions with other environmental or genetic factors.

One possible interacting environmental factor we tested was the amount of available light. Given that summer annuals emerge in sparsely vegetated spring, while winter annuals emerge in heavily vegetated fall, we expected cold temperatures followed by higher light availability to result in a higher likelihood of summer annual growth. However, results from our greenhouse experiment did not support this prediction. A systematic field survey documenting the occurrence of winter vs. summer annual horseweed across its range, along with habitat characteristics where these plants are found (e.g. mean winter temperatures, land use type and management) could suggest additional environmental factors that play a role in life cycle type differentiation and would provide key baseline data for documenting changes in proportional life cycle type occurrence over time. Studies tracking the outcomes of replicate seeds planted in fields varying in known environmental conditons would also be useful.

Overall, the plastic ability of facultative winter annuals to adjust their life cycle to suit their current seasonal environment may be adaptive. Preliminary data suggest that plants expressing the seasonally mismatched growth type (rosette in summer annual field and upright in winter annual field) have lower survival (N = 23 total flowered/247 transplanted seedlings). A highly flexible life cycle within populations may be particularly beneficial in crop fields that are actively managed to prevent horseweed growth.

Gibberellic Acid regulation may be a mechanism for horseweed life cycle type differentiation

Alongside our tests of genetic and environmental factors influencing horseweed life cycle type differentiation, we also sought to test a hypothesized mechanism by which such differentiation may occur. In line with our predictions, exogenous application of the growth hormone Gibberellic Acid (GA) increased summer annual characteristics. Also as predicted, this GA effect was stronger in unvernalized plants, while the effects of seed vernalization (in cases where it also increased summer annual characteristics) were stronger in plants not receiving GA. These results suggest that upregulation of Gibberellic Acid may be an important mechanism by which plants plastically respond to experiencing a period of cold prior to germination. It remains unclear why we did not find the predicted effect of seed vernalization and its interaction with GA for height growth rate in the growth chamber experiment, which was one of seven measures of life cycle type across both experiments. One possibility relates to the germination of many seeds during seed vernalization at 4ºC, which was surprising given prior studies reporting base germination temperatures of 8-14ºC (Steinmaus et al. 2000, Tozzi et al. 2014) and the fact that it never occurred in the common garden or greenhouse experiment. Although results were similar when excluding plants that emerged in the cold, all or a larger fraction of seeds may have begun the germination process, changing their development. We note that the height growth rates for these plants were very low in comparison with the greenhouse plants (mean = 0.1 vs. 2.0 mm/day), potentially indicating stunted growth.

It has long been hypothesized that GA and vernalization act through a common mechanism, given the ability of exogenous GA to initiate flowering in plants that normally require vernalization (Zeevaart 1983). For example, the native winter annual ecotype of *Raphanus raphanistrum* could be induced to flower without rosette vernalization by exogenous application of GA, whereas the weedy summer annual ecotype showed little response to GA (Garrison 2022). This mechanism has been studied in detail in *Arabidopsis thaliana*, where vernalization and GA both act on the floral integrator gene SOC1 (Moon et al. 2003). Syntheses on the interactive effects of temperature and GA do not generally distinguish between a cold period at the seed versus the seedling stage, although Chouard (1960) reported that GA seed treatment cannot replace seed vernalization. Our study extends prior work by suggesting that horseweed seeds experiencing cold winter conditions upregulate GA production, helping to stimulate development as a summer annual. Interestingly, applying GA at the seedling rather than at the seed stage was generally more effective in producing plants with summer annual traits. A similar tendency for GA spray compared with seed soak to cause accelerated life cycle traits has been found previously in three annual crops (Chakravarti 1958, Wilson 1981). This indicates that although the key environmental cue of cold temperatures may be experienced as a seed, the seedling stage may be a critical period for hormonal regulation. Further studies in other facultative winter annual species are needed to determine the generality of a GA-mediated seed vernalization mechanism for life cycle differentiation. In addition, future studies might compare endogenous levels of GA in plants of different life cycle types growing in the field to test the prediction that summer annual plants will show higher levels of endogenous GA. Finally, comparing the life cycle characteristics of GA-deficient mutants with control plants subjected to multiple vernalization treatments would more directly link GA upregulation to the summer annual life cycle.

Applied significance

A review of 19 common Canadian winter annual agricultural weeds found that the facultative ability to adopt a winter or summer annual life cycle predominates in these species, yet the authors noted a general lack of information on many important aspects of their biology (Cici and Acker 2009). Gaining a better understanding of the genetic and environmental factors contributing to variation in horseweed life cycle types has implications for the management of this widespread and problematic agricultural weed. According to the most recent surveys of US and Canadian farmers from the Weed Science Society of America, horseweed is among the top five most troublesome weeds in winter cereal grains, fruits, nuts, and soybeans (Van Wychen 2022, Van Wychen 2023). Of particular note for management, summer annual type plants, and especially those of resistant biotypes, were found to be more resistant to glyphosate when grown in a greenhouse (Schramski et al. 2021). This increased resistance was partially attributable to reduced glyphosate retention (Fisher et al. 2023). Prior studies in horseweed and other facultative winter annual populations have also found that by skipping the overwintering stage, spring-emerging summer annuals are more likely to survive to seeding but produce fewer seeds on average than fall-emerging winter annuals (Regehr and Bazzaz 1979, Marks and Prince 1981, Sans and Masalles 1993). Our study indicates that horseweed plants expressing either life cycle type may plastically switch to the other type in the next generation, potentially completing two generations per year. Therefore, switching crop life cycle or eliminating only those horseweed plants with the same life cycle type as the desired crop are unlikely to prevent horseweed infestation. Manipulating the winter temperatures or levels of GA experienced by horseweed seeds may allow managers to bias life cycle type differentiation in a favorable direction. Recently observed increases in the summer annual type in Michigan are less likely to be due to rapid evolution than to a change in the environment or the differential survival of the two life cycle types.

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