Germination represents a key life-history transition in plants, and timing of germination can be expected to be under strong selection in most systems. This is because timing of germination affects fitness both directly, by determining conditions during seedling establishment (Kalisz, 1986; Miller et al., 1994; Lu et al., 2016); and indirectly, by affecting other fitness-related traits such as flowering phenology (Evans and Cabin, 1995; Weinig, 2000; Donohue, 2002; Burghardt et al., 2015, 2016). In seasonal environments, germination time needs to match seasonal changes in environmental conditions influencing probability of seedling establishment and growth. Optimal timing of germination and the steepness of the fitness function are expected to differ among populations occurring in contrasting environments because of differences in the timing, magnitude, and rate of seasonal changes. For example, in autumn-germinating species, excessively high temperatures and periods of drought may prevent successful seedling establishment in summer, and optimal timing of germination should depend on when conditions become cooler and water predictably available to establishing seedlings in the autumn. Moreover, the strength of selection on timing of germination should increase with the magnitude and rate of change in environmental conditions from summer to autumn. Although spatial variation in the strength of selection on timing of germination has been documented in several systems (e.g., Kalisz, 1986; Biere, 1991; Kelly, 1992), there is a scarcity of studies experimentally examining the fitness consequences of timing of germination and their correlation with seasonal changes in temperature and soil moisture.
In seasonal environments, timing of germination is expected to be under conflicting selection through different components of fitness. Early germination is expected to be associated with large plant size, because it allows for a long period of growth before reproduction (Donohue et al., 2010; Mercer et al., 2011). In winter annuals (annuals germinating in the late summer or autumn and flowering the following year), early germination may, in addition, often be associated with favorable conditions for growth during early seedling development (relatively high temperature and long days; Kalisz, 1986; Donohue et al., 2005b; Pyl et al., 2012; Akiyama and Ågren, 2014). Because plant size is typically positively correlated with adult survival and fecundity, early germination should be favored by selection through these components of fitness (Donohue et al., 2005b; Verdú and Traveset, 2005; Mercer et al., 2011). On the other hand, early germination may be associated with low seedling survival because of recurrent drought spells or otherwise unfavorable conditions for seedling establishment at the beginning of the germination period (Donohue et al., 2005b; Gremer et al., 2020). Finally, because germination time may often affect the expression of traits later in the life cycle, the effects of timing of germination on fitness will depend also on the direction and strength of selection on these correlated traits (Weinig, 2000; Donohue, 2002; Donohue et al., 2005a; Mercer et al., 2011). A full understanding of the adaptive significance of timing of germination in a given environment thus requires that overall selection is decomposed into selection through different components of fitness, and that correlations with other fitness-related traits are examined.

The effects of germination time on adult survival and fecundity might vary among genotypes because of inherent differences in traits other than timing of germination (Mercer et al., 2011). For example, the period required between germination and flowering should affect how late a given genotype can germinate and still be able to reproduce in a seasonal environment. As a consequence, the optimal germination time may vary among genotypes with inherent differences in the length of the juvenile period, but this has not been examined empirically. This is unfortunate because such variation in optimal germination time would be important to consider for an understanding of (a) the relative performance of different genotypes in natural populations and (b) the relative importance of differences in environmental conditions and in genetic composition for among-population variation in phenotypic selection on germination timing. Information about optimal timing of germination could also be important when interpreting the results of common-garden and reciprocal transplant experiments where germination of different genotypes is typically synchronized and seedlings planted on a given day (e.g., Antonovics and Primack, 1982; Schmid, 1985; Wang and Redmann, 1996; Fournier-Level et al., 2011; Ågren et al., 2013; Ferris and Willis, 2018; Wadgymar et al., 2018; Lowry et al., 2019). If optimal germination time varies among genotypes, planting date should affect their relative fitness.

Fitness functions can be characterized by documenting relationships between natural variation in traits and relative fitness. However, strong selection is expected to reduce trait variation over time (Bulmer, 1980), and both limited trait variation and strong correlations among traits may often complicate attempts to characterize fitness functions based on available phenotypic variation (Sinervo et al., 1992; Sinervo and Basolo, 1996). To increase the range of trait variation and break correlations among traits, trait expression can be manipulated experimentally (e.g., Andersson, 1982; Boberg and Ågren, 2009), and that is the approach we take in the present study.

Here, we examine in a Mediterranean climate the effects of germination time on seedling establishment, survival, fecundity, growth rate, and flowering phenology of the annual herb Arabidopsis thaliana, and how germination time affects the relative fitness of two genotypes with inherent differences in time to first flower. The study was conducted at the site of a natural A. thaliana population in central Italy, and included two accessions, one from the local population and one from a population in north-central Sweden. Both study populations have a winter-annual life cycle, but they differ in timing of life-history transitions when grown in common environments (Ågren and Schemske, 2012; Postma and Ågren, 2016, 2018; Ågren et al., 2017). Conflicting selection on timing of germination was demonstrated at the site of the Swedish population in a field experiment in which the germination times of inbred lines from the local population were experimentally manipulated (Akiyama and Ågren, 2014). Also in the Italian population, we expect conflicting selection through seedling establishment vs. adult survival and fecundity resulting in an intermediate optimal germination time, and that timing of germination will affect seedling growth rate and flowering phenology, two fitness-related traits. However, a narrower optimum for timing of germination can be expected at the Italian site, because of more drastic seasonal changes in environmental conditions during the months of natural seedling establishment. In Italy, precipitation increases markedly in autumn after a long period of summer drought, whereas in Sweden, the seasonal change in precipitation is less pronounced (Fig. 1A–D).

We determined the mode and strength of selection on germination time in the Italian population by experimentally varying timing of germination. In addition, we examined how germination time affects the relative fitness of the Italian and the Swedish genotype. We transplanted young seedlings monthly between August and December to test the following hypotheses: (1) there is an intermediate optimum timing of germination, which coincides with the peak of germination in the local population (November); (2) early germination is associated with low seedling survival, but high adult survival and fecundity; and (3) early germination is associated with rapid early growth and with early flowering. Because of the inherent difference in time between germination and flowering start, we expected (4) the relative fitness of the Swedish genotype to decrease in later cohorts because of the limited time available for fruit production before summer drought sets in. Finally, we examined whether low seedling survival and growth were associated with periods of particularly low soil moisture and/or temperature at the study site.

**MATERIALS AND METHODS**

**Study species and study site**

Arabidopsis thaliana (Brassicaceae) is an annual, highly selfing herb native to Eurasia and Africa (Durvasula et al., 2017). The lines used in the study were originally sampled in two natural populations, close to the southern and northern range limits in Europe, respectively: one in Castelnuovo di Porto in central Italy (42°07’N, 12°29’E) and the other in Rödåsen in north-central Sweden (62°48’N, 18°12’E; see Ågren and Schemske, 2012). Although both populations have a winter-annual life cycle, the timing of life-history transitions differs. In the Italian population, seeds mature and are dispersed in late April, germinate in late October–November, and flower in February–March. By comparison, in the Swedish population,
seeds matured in the greenhouse in February 2017 and stored in a refrigerator and mid-December in 2017. For the experiment, we used planted newly germinated seedlings once a month between mid-August and mid-October, flower and fruit production of both genotypes is truncated as the number of fruits produced per seedling planted (zero if the plant did not survive until reproduction).

To investigate the effect of timing of germination on rosette size, flowering time, survival, fecundity, and overall fitness, we transplanted newly germinated seedlings once a month between mid-August and mid-December in 2017. For the experiment, we used seeds matured in the greenhouse in February 2017 and stored in paper envelopes under dry conditions at room temperature until germination. Before each transplant, seeds were sown on agar, stratified for eight days at 6°C in darkness and then moved to a growth room for 11 days (20°C 16 h day and 16°C 8 h night). The germinated seedlings, which typically were developing their first pair of true leaves, were transplanted into plug trays (2 × 2 × 4 cm plugs) filled with local soil. To allow root acclimation to the soil, seedlings were watered and kept in the shade for three days before being transferred to the experimental plot. At the site, seedlings and their associated soil plug were transferred into a randomized block design (n = 10 blocks). In each block, 240 seedlings were transferred to randomized locations in a grid consisting of 260 positions with 24 replicates of each cohort × genotype combination in each block (20 positions were left unplanted). Each cohort included 480 seedlings (240 of each genotype), for a total of 2400 seedlings (1200 of each genotype) in the experiment. Seedlings were transplanted to the site on 17 August (Cohort 1), 11 September (Cohort 2), 19 October (Cohort 3), 16 November (Cohort 4), and 14 December (Cohort 5).

Survival of plants was recorded once a month from the time of transplant until fruit maturation in late April 2018 (except that no data on survival were collected in March 2018). We defined seedling survival as the proportion of seedlings that survived until 7 January (24 days after the planting of the December cohort), and adult survival as the proportion of seedlings that survived from January to fruit maturation. We quantified rosette size by recording maximum rosette diameter from pictures taken one month after transplant and at the plant census in January using the image processing program ImageJ (Schneider et al., 2012). To document flowering start, we monitored flowering status of each plant every 3–8 days (median time interval = 7 days) between 12 February and 10 April 2018 (a total of 10 censuses) and recorded the first day when at least one flower had opened. At the first census, only two plants had begun to flower, and at the last census on 10 April, the final 29 of a total of 564 flowering plants had begun to flower. At fruit maturation at the end of April, we noted the number of fruits produced by surviving plants. At that time, flower and fruit production of both genotypes is truncated as the soil dries out at the study site. Fecundity was quantified as the number of fruits produced by reproducing plants, and total fitness as the number of fruits produced per seedling planted (zero if the plant did not survive until reproduction).

Experimental manipulation of the timing of germination

To investigate the effect of timing of germination on rosette size, flowering time, survival, fecundity, and overall fitness, we transplanted newly germinated seedlings once a month between mid-August and mid-December in 2017. For the experiment, we used seeds matured in the greenhouse in February 2017 and stored in

FIGURE 1. Average climatic conditions at the sites of origin of the two Arabidopsis thaliana genotypes included in the present study. Monthly mean soil temperature averaged across the period 2003–2018 in (A) Castelnuovo di Porto, Italy, the site of the present study, and in (B) Rödåsen, Sweden. For both locations, means were calculated on hourly recordings by one to four sensors placed 1 cm below ground (for more details, see Ågren and Schemske, 2012). Mean monthly precipitation averaged across the period 1970–2000 in (C) Castelnuovo di Porto and (D) Rödåsen (data from Fick and Hijmans, 2017).

Statistical analysis

We used linear and generalized linear models to assess the effects of cohort, genotype (Italian or Swedish), block, and the cohort × genotype interaction on fitness, seedling survival, adult survival, fecundity, rosette size, and flowering start. When the cohort ×
genotype interaction was statistically significant, we analyzed the effect of genotype in models separately by cohort. For the analysis of fitness, we used a zero-inflated negative binomial model to correct for both the excessive presence of zeros and overdispersion. For the analyses of seedling and adult survival, we used a binomial error distribution, and for the analysis of fecundity a quasi-Poisson error distribution to correct for overdispersion. In all the remaining models, error was normally distributed. Statistical significance was examined using $F$-tests based on type III sums of squares. Because no seedling survived the first month after transplant in the first three cohorts, these cohorts were excluded from the statistical analyses.

To quantify selection against the nonlocal Swedish genotype, we calculated selection coefficients in the November and December cohorts ($s = 1 - (\text{mean fitness of the less fit genotype} / \text{mean fitness of the fittest genotype})$) based on total fitness and its components. The selection coefficient was given a negative sign if the nonlocal genotype outperformed the local genotype. Confidence intervals for selection coefficients were estimated by nonparametric bootstrapping (15,000 times). Fitness data were resampled with replacement from within experimental blocks. Selection coefficients were calculated for each bootstrap sample, and we estimated 95% confidence intervals as the intervals between the 2.5% and 97.5% quantiles of these samples. We tested the null hypothesis that there is no adaptive differentiation between the Italian and the Swedish genotype using two-tailed $P$-values, calculated as twice the proportion of bootstrap values overlapping zero. To assess whether selection against the Swedish genotype was stronger in the December cohort than in the November cohort, we calculated the difference between randomly paired bootstrapped estimates of selection coefficients in the two cohorts (15,000 pairs) and calculated the number of times the difference overlapped with zero, with $\leq 2.5\%$ cases implying statistical significance at $P = 0.05$ given a two-sided hypothesis. All statistical analyses were performed in R version 3.4.0 (R Core Team, 2017).

**RESULTS**

**Temperature, precipitation and soil moisture**

Climatic conditions immediately after transplant differed strongly between cohorts. Air and soil temperatures showed a steady decrease from August to December (Fig. 2A, B), from a mean of $\sim 23^\circ\text{C}$ in the August cohort to $\sim 7^\circ\text{C}$ in the December cohort during the first month after transplant. Minimum air temperatures reached close to freezing 12 days after transplant of the November cohort and five days after transplant of the December cohort (Fig. 2B). Precipitation was irregular, and the three first cohorts experienced periods of very low...
soil moisture content (less than −300 kPa) during the first month after transplant (Fig. 2C, D). In contrast, soil moisture was consistently high the first month after transplant of the November and December cohorts, never reaching lower than −24 kPa (Fig. 2D).

**Timing of germination vs. total fitness**

There was strong evidence of stabilizing selection on germination time. No seedling in the first three cohorts survived even the first month after transplant (Appendix S1), whereas seedlings transplanted in November had markedly higher fitness than seedlings transplanted in December (32 times higher in the Italian genotype, 50 times higher in the Swedish genotype; Fig. 3A and Table 1). There was no significant cohort × genotype interaction in the analysis of overall fitness (Fig. 3A and Table 1), and selection against the non-local Swedish genotype did not differ between the November and December cohorts (November cohort, s = 0.64; December cohort, s = 0.69, P = 0.42; Appendix S2).

**Timing of germination vs. survival and fecundity**

Differences in all fitness components contributed to the overall higher fitness of seedlings establishing in November compared to other cohorts, with differences in fecundity and seedling survival explaining most of the advantage of the November cohort compared to the December cohort (Fig. 3B–D). Differences in fecundity largely explained the higher fitness of the local Italian genotype compared to the nonlocal Swedish genotype.

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**FIGURE 3.** The effects of timing of germination on overall fitness, survival, and fecundity of the Italian (closed bars) and the Swedish (open bars) genotypes of *Arabidopsis thaliana* at the site of the Italian population. (A) Overall fitness (number of fruits produced per seedling planted) of the August, September, October, November, and December cohorts, respectively. (B) Seedling survival (survival until early January). (C) Adult survival (survival from early January to fruit maturation). (D) Fecundity (number of fruits per surviving plant). Means ± SE are given. No seedling survived the first month after planting in the first three cohorts, and these cohorts are therefore not included in panels C and D. Statistically significant effects of cohort, genotype, and the cohort × genotype interaction in generalized linear models are indicated (see Table 1 for details). **P < 0.01, ***P < 0.001.
In all cohorts, the highest rate of mortality was observed during the first month after transplant (Appendix S1). Seedling survival of the November cohort was more than twice as high compared to that of the December cohort (Table 1 and Fig. 3B).

Timing of germination vs. rosette size and flowering phenology

Rosette size differed between cohorts, but not between genotypes. In January (i.e., prior to any flowering), the rosette diameter of seedlings in the November cohort was 1.5 times larger than that of seedlings in the December cohort (mean rosette diameter, 12.4 vs. 8.3 mm, \( F_{1,134} = 225.0, P < 0.001 \)), but rosette size did not differ significantly between genotypes (Table 1 and Fig. 3D); and there were no significant differences between selection coefficients calculated based on rosette size (Appendix S2).

In January (i.e., prior to any flowering), the rosette diameter of seedlings in the November cohort was 1.5 times larger than that of seedlings in the December cohort (mean rosette diameter, 12.4 vs. 8.3 mm, \( F_{1,134} = 225.0, P < 0.001 \)), but rosette size did not differ significantly between genotypes (Table 1 and Fig. 3D); and there were no significant differences between selection coefficients calculated based on rosette size (Appendix S2).

Seedling survival during the first month after transplant varied significantly between cohorts and genotypes (Table 1 and Fig. 3B). The effect of genotype on seedling survival differed between the two cohorts (significant cohort × genotype interaction; Table 1). In the November cohort, seedling survival of the Swedish genotype was 1.09 times higher than that of the Italian genotype (\( F_{1,46} = 10.5, P = 0.001 \)), whereas no significant difference between genotypes was recorded in the December cohort (\( F_{1,468} = 0.20, P = 0.66 \)). Selection coefficients based on seedling survival did not differ significantly between cohorts (November cohort, \( s = −0.09 \); December cohort, \( s = 0.05; P = 0.32 \); Appendix S2).

The fecundity of the November cohort was ~10 times higher than that of the December cohort, and in both cohorts, the Italian genotype produced ~3 times more fruits than the Swedish genotype (no significant cohort × genotype interaction; Table 1 and Fig. 3C); and there were no significant differences between selection coefficients calculated based on fecundity (November cohort, \( s = 0.67 \); December cohort, \( s = 0.68; P = 0.89 \); Appendix S2).
first month in the December compared to the November cohort. Of the two cohorts that survived the first month after transplant, the November cohort had the highest adult survival and fecundity, and these differences were associated with size differences established early in plant development. A similar strong effect of germination time on growth rate early during seedling establishment and final rosette size of *Arabidopsis thaliana* has been documented in field experiments conducted in northern Europe (Akiyama and Ågren, 2014) and North America (Donohue, 2002). The difference in rosette size was associated with a difference in flowering time. The November cohort flowered 28 days earlier than did the December cohort (Fig. 4B), and previous work has shown that early flowering is strongly favored by selection at this site (Ågren et al., 2017). The results are consistent with the idea that timing of germination affects fitness both directly, because of effects on seedling survival and growth, and also indirectly, because of effects on flowering time (Donohue, 2002; Donohue et al., 2005a; Burghardt et al., 2015, 2016).

In contrast to the present study, most previous studies of selection on germination timing have found evidence of directional selection for earlier germination (Kalisz, 1986; Donohue, 2002; Donohue et al., 2005b; Verdú and Traveset, 2005; Akiyama and Ågren, 2014). One likely reason is that in these studies the range of germination time examined has been restricted to the period of natural germination in the local population, which might have prevented the discovery of an intermediate optimum. As for other traits (e.g., Nilsson, 1988; Dudley and Schmitt, 1996; Galloway and Burgess, 2009; Cuartas-Domínguez and Medel, 2010), an experimental increase in the magnitude of variation in germination time allowed a more complete characterization of the shapes of fitness functions in the present study.

The local Italian genotype had higher fitness than the nonlocal Swedish genotype in both the November and December cohorts (Fig. 3A). The strong selection observed against the nonlocal genotype is consistent with the results of previous reciprocal transplant experiments between these two populations (Ågren and Schemske, 2012; Ågren et al., 2013; Postma and Ågren, 2016). The fitness advantage of the Italian genotype was mainly due to its higher fecundity (Fig. 3D), which was associated with its earlier start of flowering. Because the time between germination and flowering start is inherently longer in the Swedish compared to the Italian genotype and the period available for flower and fruit production is strictly limited by drought in spring, we expected selection against the nonlocal Swedish genotype to be strongest in the last cohort. However, the relative fecundity of the Italian genotype compared to the Swedish genotype was not greater in the December than in the November cohort. One reason could be that the difference in flowering time was only half as large in the December cohort (12 vs. 25 days). This shows that although the two genotypes differ in the time
required between germination and flowering, the magnitude of this difference is affected by the environment to which seedlings are exposed, and the difference in time available for flower and fruit production before spring drought set in was smaller in the December than in the November cohort. Strong environmental effects on the magnitude of the difference in flowering time of these two genotypes have previously been documented in reciprocal transplants between their native sites (Ågren et al., 2017).

Interestingly, seedling survival of the Swedish genotype was 9% higher than that of the Italian genotype in the November cohort, which to some extent counteracted the strong selection against the nonlocal Swedish genotype through fecundity. The two genotypes do not show constitutive freezing tolerance, but they differ in their ability to acclimate in response to cold, nonfreezing temperatures (Swedish genotype > Italian genotype; Oakley et al., 2014). The time between planting and exposure to near-freezing temperatures was longer in the November than in the December cohort (Fig. 2A, B), which may have allowed the Swedish genotype to develop a greater freezing tolerance in the former cohort. Our results suggest that the effect of germination timing on survival is contingent on the ability of a given genotype to acclimate to low temperatures.

The shape of the relationship between timing of germination and fitness differed between the present study and a previous study conducted at the site of the Swedish genotype, where seedlings of the local population were transplanted once a month between August and October (Akiyama and Ågren, 2014). The optimum germination timing was November in Italy and August in Sweden, and the time window available to seedling establishment was much shorter in the present study than observed in Sweden (Akiyama and Ågren, 2014). In addition, differences in fitness among cohorts were markedly larger in Italy: the ratio between mean fitness of the best and that of the second-best-performing cohort was ~4.5 at the Swedish site and 32 at the Italian site. The stronger selection on germination timing in Italy compared to Sweden is likely related to the steep fitness function and the narrowly defined optimal timing of germination at the Italian site.

The magnitude of the difference in flowering time of these two genotypes was markedly larger in Italy: the ratio between mean fitness of the best and that of the second-best-performing cohort was ~4.5 at the Swedish site and 32 at the Italian site. The stronger selection on germination timing in Italy compared to Sweden is likely related to the steep fitness function and the narrowly defined optimal timing of germination at the Italian site.

CONCLUSIONS

We found evidence of strong stabilizing selection on timing of germination in a Mediterranean natural population of *Arabidopsis thaliana*. Selection on germination time was markedly stronger in our study population compared to another natural population located in Sweden, where precipitation and soil moisture change less and more slowly during late summer and autumn. Spatial variation in the strength of selection on timing of germination (e.g., Kalisz, 1986; Biree, 1991; Kelly, 1992), and on phenological traits in general, have been documented in a wide range of both plant and animal species (see Siepielski et al., 2013). Differences in temperature and soil water availability have been implied to explain variation in selection on phenological traits such as flowering time (e.g., Maad, 2000; Giménez-Benavides et al., 2011), but experimental tests of such hypotheses are still rare. The results of the present study suggest that both the rate and magnitude of seasonal changes in environmental conditions should be considered as important determinants of variation in selection on phenological traits.

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AUTHOR CONTRIBUTIONS

G.Z., M.V., and J.A. designed the study. G.Z. and M.V. collected the data. G.Z. analyzed the data. G.Z. and J.A. wrote the manuscript. All authors gave final approval for publication.

DATA AVAILABILITY

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.5qftdz2m (Zacchello et al., 2020).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Survival curves of the five germination cohorts.

APPENDIX S2. Selection coefficients against the nonlocal Swedish genotype in the November and the December cohort.

APPENDIX S3. Effects of germination cohort, genotype, the cohort × genotype interaction and block on rosette diameter one month after transplant and in January, and on flowering time.

APPENDIX S4. Effects of timing of germination on rosette diameter one month after transplant.

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