Combining the resurrection approach with transplant experiments to investigate adaptation of plant populations to environmental change

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Abstract

Recent climatic changes, such as more frequent droughts and heatwaves, can lead to rapid evolutionary adaptations in plant populations. Such rapid evolution can be investigated using the resurrection approach by comparing plants raised from stored ancestral and contemporary seeds from the same population. This approach has so far only been used in common garden experiments, allowing to reveal genetic differentiation but not adaptation. In this study, we performed a novel approach by testing for evolutionary adaptation in natural plant populations using a resurrection study in combination with *in situ* transplantations. We cultivated seedlings from ancestors (23-26 years old) and contemporary descendants of three perennial species (*Melica ciliata*, *Leontodon hispidus* and *Clinopodium vulgare*) from calcareous grasslands in the greenhouse and transplanted them back to their collection sites. In addition, we sowed seeds of ancestors and descendants of two species (*L. hispidus* and *C. vulgare*) to the collection sites in order to investigate germination rates. In transplanted *M. ciliata* seedlings, we observed lower mortality, larger plant size and more leaves in descendants compared to ancestors. This indicates that descendants are better adapted than ancestors to the current environmental conditions, which proved to be exceptionally hot and dry during the study period. Descendants of *C. vulgare* seedlings tended to be smaller and descendants of *L. hispidus* seedlings produced less leaves compared to their ancestors in their contemporary environmental conditions. In *C. vulgare* and *L. hispidus*, we found evolution towards faster germination, and especially descendant seeds of *C. vulgare* were better adapted to the unfavourable conditions during the experimental period. Concluding, we demonstrate that our novel approach to combine resurrection ecology with transplant experiments is a promising avenue to rigorously test for evolutionary adaptations in changing environments.

1. Introduction

Environmental conditions are rapidly changing since the last decades and will continue to change in the future (IPCC, 2018). In North-western Europe, precipitation is expected to decrease, especially in summer, and evapotranspiration will increase due to higher temperatures. Together, these will lead to higher frequencies and intensities of droughts (Dore, 2005; Ruosteenoja et al., 2018; Samaniego et al., 2018; Spinoni et al., 2018). Given these ongoing changes, understanding and predicting the capacity of natural plant populations to evolve rapidly to such changing environmental conditions is a high priority in current and recent research (Exposito-Alonso et al., 2018; Franks et al., 2018).

Over the last two decades, the resurrection approach has been applied as a powerful method to study evolutionary changes of plant populations to recent global change (Franks et al., 2018; Kooyers et al., 2021; Vtipil and Sheth, 2020). This approach consists of an experimental design that uses seeds from a population collected before (ancestors) and after (descendants) a potential selection pressure (*e.g.* consecutive years of drought, changes in nutrient supply, etc.). Comparing phenotypes of these two generations in a common environment may then reveal evolutionary changes (Franks et al., 2007). Recently, seeds collected for storage in seed repositories have been successfully used in resurrection studies (Ensslin et al., 2023; Everingham et al., 2021; Rauschkolb et al., 2022a, 2022b). Resurrection studies convincingly showed that evolution of plant populations can occur rapidly within only a few generations (Franks et al., 2007; Hamann et al., 2018; Thompson et al., 2013). Rapid evolutionary changes have been documented in morphological, phenological, and physiological trait changes in response to drought stress (Franks et al., 2007; Nevo et al., 2012; Sekor & Franks, 2018; Thomann et al., 2015; Vigouroux et al., 2011). Many resurrection studies using predominantly annual species reported evolution of earlier onset of flowering after a few years of intense droughts (Franks et al., 2007; Nevo et al., 2012; Thomann et al., 2015). In some species, seeds also emerged more rapidly and showed earlier flowering and smaller plant size (Kulpa and Leger, 2013; Dickman et al., 2019; Sekor and Franks, 2018). These evolutionary changes are consistent with a drought escape strategy involving rapid development to complete the life cycle while resources are still available (Basu et al., 2016).

To date, the resurrection approach has mainly been applied in common garden experiments located in a greenhouse, in *ex situ* experimental plots or in growth chambers (Thomann et al., 2015; Vtipil & Sheth, 2020; Wooliver et al., 2020; Rauschkolb et al., 2022a, 2022b). As an interesting extension to resurrection studies, researchers have included treatments or multiple environments to study whether, in addition to genetic differentiation of trait means, the evolution of phenotypic plasticity has potentially been mediated by specific environmental drivers (Blanquart et al., 2013; Rauschkolb et al., 2022a, 2022b). However, the artificial conditions do not fully mirror those of the natural environment in which the populations have evolved. For instance, temperature, water and nutrients are often much more benign under controlled conditions (Poorter et al., 2016). Moreover, temporal dynamics of air temperature and soil water availability, typical in natural environments, are very difficult to realistically implement under experimental conditions (Poorter et al., 2016). Thus, these experiments cannot deliver firm proof of adaptive evolution, since we cannot be sure how the fitness of plants in *ex situ* cultivation relates to fitness in field conditions (Kawecki and Ebert, 2004). To convincingly demonstrate local adaptation, reciprocal transplant experiments remain the gold standard (Kawecki & Ebert 2004, Blanquart et al 2013), and a logical step is therefore to combine resurrection studies with *in situ* transplantations in the sites of population origins (Franks et al. 2018), but to our knowledge no such studies have been published so far.

Reciprocal transplant experiments are a powerful tool to detect adaptation and investigate fitness trade-offs by transplanting plants from different populations reciprocally to all respective origins (Kawecki & Ebert, 2004; Fig. 1). This method can be partially applied to the resurrection approach by transplanting ancestors and descendants to the exact location where they were sampled (*in situ* transplantation; Fig. 1). By doing so, the strength of adaptation of the ancestral and descendant populations to the current (but not to the past) environment can be measured. Ensslin et al. (2023) already performed a similar method comparing plants in the field from stored seed bank material, from *ex situ* cultivation and from a sampled wild population close to the original population. However, their study did not focus on investigating evolutionary change, but rather the effect of *ex situ* cultivation on germination and establishment of plants reintroduced into the wild.

Here, we investigated recent adaptive evolution of three plant species from calcareous grasslands in Belgium by conducting a resurrection study in combination with *in situ* transplantations. We used ancestors sampled 23-26 years ago and contemporary descendants of a population of *Melica ciliate, Clinopodium vulgare* and *Leontodon hispidus*. We transplanted seedlings and sowed seeds from ancestors and descendants to the original sampling location, we measured mortality, plant size and number of leaves on the seedlings, and we observed germination of the seeds. We hypothesized that descendants show stronger adaptation than their ancestors in terms of higher germination rate, better plant performance and lower plant mortality under current conditions due to two decades of adaptation to environmental changes. Finally, we discuss the conceptual integration of *in situ* transplantations into the resurrection approach.

2. Material and methods

2.1. Study species

We selected three perennial plants that are typical species in calcareous grasslands and widespread across Europe: *Melica ciliata* L. (Poaceae), *Clinopodium vulgare* L. (Lamiaceae) and *Leontodon hispidus* L. (Asteraceae). *Melica ciliata* flowers in June, *C. vulgare* flowers from July to September and *L. hispidus* flowers from June to October (Kühn and Klotz, 2002). All selected species are hemicryptophytes and part of the floristic composition of semi-natural dry grasslands. This habitat is considered as a conservation priority by the European Commission ("Festuco-Brometalia"; EU code 6210: Semi-natural dry grasslands and scrubland vegetation on calcareous substrates).

2.2. Seed origin

For each species, we chose one population in a calcareous grassland located in Belgian nature reserves (Fig. 2, Table S1). The populations are relatively isolated and gene flow between other populations should be minimal. The distance to the nearest population of the same species is 1.08 km for *M. ciliata*, 4.09 km for *C. vulgare*, and 1.86 km for *L. hispidus*. Seed material of the ancestors were collected in the years 1992-1995 and seed material of the descendants in 2018-2020 depending on the species. *Clinopodium vulgare* and *L. hispidus* can reproduce in the first year, while *M. ciliata* typically reproduces in the second year, which means there may have been up to 23 generations for *C. vulgare*, 26 generations *L. hispidus* and up to 15 generations for *M. ciliata* between the collection years. Ancestral seed collections were performed by the seed bank staff of Meise Botanic Garden (Belgium) for conservation purposes. The botanical garden reported that the number of sampled individuals was maximized to represent the population, but exact numbers were not recorded. All seeds were cleaned, bulked, dried at 15 % relative humidity, and stored at -20 °C at the seed bank of the Meise Botanic Garden. In the summer of 2018, seeds from 20-47 mother plants of *C. vulgare* and *L. hispidus* were collected from the exact same populations (Table S1). Those seeds were cleaned, bulked and then stored at 4 °C. Rauschkolb et al. (2022a) showed that genomic relatedness of the ancestors and descendants of these populations is similar, which supports that sampling procedures were comparable and a sufficient number of seeds was collected. Seeds from *M. ciliata* were collected in 2020 by the Meise Botanic Garden using the same sampling protocol as applied in 2018.

We obtained meteorological data from the Royal Meteorological Institute of Belgium for the weather station “Dourbes”, which is located 3 km from the *M. ciliata* population and 11 km from the *C. vulgare* population, as well as from the Royal Netherlands Meteorological Institute for the weather station “Maastricht”, which is located 14 km from the *L. hispidus* population. The meteorological data contains daily maximum and mean temperature as well as daily precipitation from 1966 until 2022. We calculated the mean and maximum temperature and total precipitation for each month during the growth season from March to August of 2022 as well as the 30-year-average (1991-2021). These data were used to compare the weather conditions during the year of study with the long-term average. Furthermore, we used the homogenized data from the “Dourbes” weather station to calculate the temperature anomaly for each year as the difference between its average annual temperature and the average temperature from 1966 until 2020 (Fig. S2 B). Following Liao et al. (2020), we additionally calculated the climatic water deficit (CWD) for this area, which is an estimate of drought stress in plants. CWD is quantified as the amount of water by which potential evapotranspiration exceeds actual evapotranspiration (Stephenson, 1998). Solar radiation data was retrieved from Hargreaves (1994). The temperature anomaly steadily increased from 1966 until 2020 and mean temperature in the seed collection years was 9.41 °C in 1992 (ancestors) and 10.25 °C in 2018 (descendants; Fig. 2S B). The average CWD steadily increased during the period from 1966 to 2020 and was 66 % higher in 2018 compared to 1992 (Fig. 2S A). The focal populations were thus exposed to a progressively diminishing water availability in response to increasing temperatures between sampling of ancestors and descendants.

2.3. Experimental design

To reduce environmental, maternal and storage effects (Franks et al., 2007), a refresher generation of both ancestors and descendants (hereafter referred as ‘temporal origins’) was grown under standardised greenhouse conditions (Rauschkolb, et al., 2022b). We sowed 200-300 seeds of each temporal origin and 15 seedlings were randomly selected and cultivated for each temporal origin. We kept the plants from the two temporal origins in separate cages to prevent unintentional cross-pollination (Rauschkolb, et al., 2022b). Emerging flowers were randomly pollinated by hand using pollen from individuals of the same temporal origin. Seed production of *L. hispidus* was too low for the experiment, hence another refresher generation was grown using the same protocol, but this time bumblebees (Natupol Seeds, Koppert GmbH) were used for pollination. The refresher generation failed for *M. ciliata*, because the plants did not flower, so for this species we used seeds without a refresher generation in our experiments.

In September 2021, refreshed seeds from 12 seed families per temporal origin of *C. vulgare* and *L. hispidus* and from 14 seed families of *M. ciliata* descendants were sown in trays with cultivation soil (Anzuchterde, Hawita Fruhstorfer Erde) to produce 14 seedlings per seed family. For ancestors of *M. ciliata*, we sowed 100 random seeds from the ancestral seed bulk, which resulted in 60 plants available for the transplantation. After germination, seedlings were singled out and transplanted to 54-multitray-pots (Meyer KG) with standard potting soil (Type T1b, Hawita Fruhstorfer Erde). The seedlings were kept in the greenhouse of the “Wissenschaftsgarten” at Goethe University Frankfurt. Three days prior to transplantation (October 2021) we measured plant size variables: height of the longest stem for *C. vulgare* and *M. ciliata* and rosette diameter for *L. hispidus*. After the measurements the plants were moved outdoors for cold acclimation. To avoid confusion with other individuals of the same species, we set up a plot for transplantation in the vicinity of the original population where no natural individuals were present. The distance from the plot to the edge of their natural population is 295 m for *M. ciliata*, 15 m for *C. vulgare* and 105 m for *L. hispidus.* The plots for *L. hispidus* and *M. ciliata* were situated on flat ground while the plot for *C. vulgare* was situated on a slope. To prevent herbivory and trampling by large grazers, each plot was fenced. We cut the standing vegetation with a brushcutter to facilitate transplantations. The plots were divided into 12 blocks for *C. vulgare* and *L. hispidus* and into 10 blocks for *M. ciliata*. The blocks were 1 m × 1.5 m wide and contained 24 individual plants in 4 columns and 6 rows separated by 25 cm. Plants were distributed among the blocks equally according to temporal origin and seed family, but randomized within each block. In February 2022, we visited the plots to sow seeds from *C. vulgare* and *L. hispidus.* We collected local soils within the plot and sieved it through a 5 mm mesh size. The sieved soil was used to fill six 54-multitray-pots (Meyer KG), which were placed with their surface at ground level. For each seed family of ancestors and descendants, we sowed ten seeds into twelve pots (replicates). The replicates were distributed equally among the multitrays (blocks) and then randomized in each tray. After sowing, the trays were covered with a fine white net curtain to prevent seed spillover and herbivory interference.

In the center of each plot, one data logger (iButton DS1923, Maxim Integrated) was positioned to record air temperature and relative humidity every four hours over the whole experimental period. Additionally, two data loggers (iButton DS1921G-F5, Maxim Integrated) were buried 5 cm deep in the center of two opposing corner blocks to measure soil temperature every 4 hours. Furthermore, in order to make sure that soil conditions were comparable, we took four random 25 cm^2 soil samples from each transplantation plot and from within the native population site at 10 cm depth. The four samples from each location were mixed together and dried at 40°C for one week. The samples were sieved to < 2 mm, and 0.3–1g of sieved soil was milled with the Mixer Mill MM400 (Retsch, Haan, Germany) for 60 seconds with 30 rounds per second. To avoid contamination between samples, the sieving and milling tools were cleaned between samples with an air-compressor and water. The chemical composition of the samples was analyzed to determine the amount of fundamental minerals for plant development (*i.e.*, plant-available P, K, S, Ca and Mg, total element content of P, K, S, Ca, total C, N and S), as well as pH level and salinity (Table S4).

2.4. Measurements in the field

The plots were revisited in April 2022 and we measured the following: germination, mortality, number of leaves and plant size. In July 2022, we only visited the *M. ciliata* plot due to time constraints and recorded mortality and plant size. We visited all plots in August 2022 and recorded germination, mortality, and plant size. In April 2023, we only recorded germination at the *C. vulgare* and *L. hispidus* plots.

2.5. Data analysis

All analyzes were conducted using R (version 4.0.3, R Core Team, 2020). To compare *M. ciliata* ancestors (60 plants, seed families unknown) and descendants (180 plants from 14 seed families), we created a completely random subset of the descendants consisting of 60 plants. No significant differences ($p < 0.05$) in plant size and number of leaves were found between the randomly selected subset of descendants and the complete dataset (Table S2), and we therefore used the subset for further analysis.

We applied generalized linear mixed-effects models (GLMM) with binomial family implemented in the *lme4* package (Bates et al., 2015) to analyze differences in mortality (response variable) between temporal origins (*i.e.*, ancestors vs. descendants). Temporal origin, time (*i.e.*, the different times when plants were measured) and their two-way interaction were included as fixed factors, and seed family (for data of *C. vulgare* and *L. hispidus*) and block as random factors.

We used linear mixed-effects models (LMMs, *lme4* package) to test for differences in morphological traits between temporal origins at several measurement times. Plant size and number of leaves were included as response variables in separate models. For plant size, we included temporal origin, time and their two-way interaction as fixed factors, and seed family (for data of *C. vulgare* and *L. hispidus*), block and individual (to account for multiple measurements over the same individual) as random factors. For number of leaves, the same model was used, but we additionally included initial size as covariate to control for potential differences in size of the transplanted seedlings.

We also analyzed whether died plants had a different plant size from plants that survived in the previous measurement using LMMs. To this aim, we included plant size as response variable and mortality at the next measurement, temporal origin and their two-way interaction as fixed factors, and seed family (for data of *C. vulgare* and *L. hispidus*), block and individual (for data of *M. ciliata*) as random factors. We used a logistic regression to confirm the results of this model (GLMM, binomial family) with mortality as the response variable and time, plant size at the previous measurement and their interaction as explanatory variables. We also included seed family (*C. vulgare* and *L. hispidus*) and block as random factors.

In addition, we used generalized linear mixed-effects models (GLMM) with Poisson family implemented in the *lme4* package (Bates et al., 2015) to analyze differences in the number of germinated seeds (response variable) between temporal origins. Temporal origin, time and their interaction were included as fixed factors and block, seed family nested in block, and individual as random factors. We checked the model for zero inflation using the function "check_zeroinflation" from the *performance* package (Lüdecke et al., 2021). The model for *C. vulgare* was underfitting zeros (ratio of 0.80), hence we used a negative binomial GLMM from the *lme4* package (Bates et al., 2015) instead.

In all LMMs, the assumptions of normality and homogeneity of variance of the residuals were tested using the Shapiro-Wilk test and visually checked through plotting the fitted versus residual values. If needed, the response variables were log- or square-root-transformed to meet the parametric assumptions. Whenever time of measurement or the temporal origin × time of measurement interaction was significant, we applied post-hoc contrasts using Tukey's test from the *emmeans* package (Lenth, 2021).

3. Results

In *M. ciliata* mortality did not differ between temporal origins in April 2022, but was significantly higher in ancestors compared to descendants in July 2022 (55.0 % mortality in ancestors and 20.0 % mortality in descendants, Fig. 3A) and in August 2022 (78.3 % *versus* 56.7 % mortality, respectively, Fig. 3A). Mortality was significantly affected by temporal origin and time of measurement ($p < 0.001$, Table 1), but not by their interaction ($p = 0.804$, Table 1). Plant size significantly differed depending on mortality at the next measurement ($p < 0.001$, Table 1) and, vice versa, mortality differed depending on the plants size at the previous measurement (Table S3), meaning that *M. ciliata* plants that would not survive until the next census were generally smaller than the plants that survived (Fig. 4A). Moreover, plant size was significantly affected by temporal origin, time of measurement and their interaction ($p < 0.001$, Table 1). Descendants were bigger compared to the ancestors at all measurement times (Fig. 3B). Furthermore, there were no significant differences in plants size of descendants between October 2021 and April 2022, whereas ancestors grew significantly by 1.49±0.12 cm (Mean±Standard Error, Fig. 3B) during this time. In April 2022, descendants carried 6.65±0.47 leaves, which was significantly more than ancestors which had 4.75±0.34 leaves ($p = 0.002$, Fig. 3C, Table 1).

In *C. vulgare*, mortality did not differ between temporal origins in April 2022 ($p = 0.339$, Table 1) and reached 100% in August 2022 for both temporal origins (Fig. 3D). Plant size was marginally significantly affected by mortality ($p = 0.092$, Table 1), but post-hoc tests did not reveal significant differences between alive and dead plants ($p > 0.05$, Fig. 4B) and the logistic regression found no significant effect of plant size on mortality ($p = 0.207$, Table S3). However, plant size was significantly affected by time of measurement ($p < 0.001$, Table 1) and the interaction of time of measurement and temporal origin ($p = 0.006$, Table 1). Plant size and number of leaves were not significantly different between ancestors and descendants ($p > 0.05$, Table 1, Fig. 3EF). From the seeds we sowed in the experimental plot, we recorded germination neither in April 2022 nor in August 2022 regardless of the temporal origin (Fig. 5A). In April 2023, however, we recorded significantly more seedlings of descendants (3.69±0.17 seedlings per pot, Fig. 5A) compared to ancestors (3.31±0.18 seedlings per pot, Fig. 5A, $p = 0.03$, Table 2).

In *L. hispidus*, mortality did not differ between temporal origins ($p = 0.316$, Table 1) and reached 100% in August 2022 for both temporal origins (Fig. 3G). Plant size tended to differ between dead and alive plants ($p = 0.058$, Table 1) and plant size significantly affected mortality according to the logistic regression ($p < 0.01$, Table S3). However, post-hoc tests did not reveal significant differences between alive and dead plants ($p > 0.05$, Fig. 4C), but surviving plants tended to be bigger than those that would die. Plant size was significantly affected by temporal origin ($p = 0.013$, Table 1), time of measurement ($p < 0.001$, Table 1) and their interaction ($p = 0.035$, Table 1). Descendants were 1.18 cm bigger than ancestors in October 2021, but had the same size as ancestors in April 2022 (Fig. 3H). Number of leaves was significantly higher in ancestors (7.77±0.25, Fig. 3I, $p = 0.002$, Table 1) compared to descendants (7.03±0.16, Fig. 3I) in April 2022. Regarding the seed sowing experiment, there is a significant interaction of temporal origin and time in terms of the number of recorded seedlings ($p = 0.013$, Table 2). In April 2022 we recorded significantly more seedlings per pot of descendants (0.43±0.09 seedlings, Fig. 5B) compared to ancestors (0.22±0.06 seedlings, Fig. 5B, post-hoc test: $p = 0.0084$). At the next census in August 2022, no seedlings were recorded for both descendants and ancestors, while in April 2023 new seedlings had emerged but descendants (0.36±0.07 seedlings, Fig. 5B) and ancestors (0.45±0.10 seedlings, Fig. 5B) did not significantly differ in this respect (post-hoc test: $p = 0.5955$).

4. Discussion

In this study, we incorporated transplantation experiments into the resurrection approach to study adaptive evolution of three populations of different plant species to recent environmental change. We detected evolutionary changes in all three study species. We found greater plant size, more leaves and lower mortality in descendants of *M. ciliata* compared to their ancestors. Descendants of *C. vulgare* tended to produce a lower plant size and slightly more germination in field conditions compared to their ancestors. In *L. hispidus*, we found a larger plant size and less leaves in the juvenile stage of the descendants compared to their ancestors. We also observed a higher percentage of germinated seeds in *C. vulgare* and *L. hispidus* in descendants compared to ancestors.

4.1. Genetic differentiation between descendants and ancestors in their natural environment

In the first year of the experiment, the germination of the seeds we sowed in the field was very low. We did not record any seedlings of *C. vulgare* in 2022, which was most likely driven by the extremely low water availability during this year. Seeds of *L. hispidus* managed to germinate in April 2022 and we recorded more descendant seedlings (4.3 % of seeds sown) compared to ancestors (2.2 % of seeds sown). Thus, descendant seeds seem to be able to germinate faster or need less water to germinate. However, all seedlings died before the next census in Autumn 2022 irrespective of temporal origin. Hence, in our experiment the faster germination of the descendant seedlings did not lead to a drought escape (Basu et al., 2016), because all plants died before they could reproduce. We have to consider, that our experimental setup does not fully represent natural conditions. If the seeds would have germinated on the natural soil – i.e. not in multitrays with local soil – in the natural vegetation – i.e. not clipped to facilitate the transplantation –, the faster germination might have helped the seedlings to grow deeper roots to reach soil layers with higher water content in a potentially more benign aboveground microenvironment. In April 2023, some seeds of *L. hispidus* germinated, but there were no significant differences in germination rate between descendants and ancestors. The conditions in April 2023 were much more favourable compared to the previous year, but the germination rate of *L. hispidus* was still similar to April 2022. This might be due to high seed mortality during 2022 or mortality of seedlings that germinated in between our measuring visits.

In contrast, we recorded a high number of seedlings of *C. vulgare* in April 2023, with more seedlings of descendants (36.9 % of seeds sown) compared to ancestors (31.3 % of seeds sown). These germination rates are in the same range of what we have observed under greenhouse conditions, which usually fluctuate around 45 %. The higher seedling count of the descendants can have multiple causes. The population might have evolved a higher longevity in the dormant state leading to more viable seeds (Dalling et al., 2011). Descendant seeds might also require less water for germination or have more efficient water uptake to facilitate germination (Baskin and Baskin, 2015). It is also possible that less descendant seedlings germinated and subsequently died before we could document their germination. Even though we cannot pinpoint the underlying cause with our data, the findings show that the descendant seeds are better adapted to the contemporary environmental conditions.

Mortality rates were high for transplanted plants of all species in 2022, which impeded the follow-up of the entire life cycle of the study species. This was very likely caused by the extreme temperatures and drought (*e.g.*, soil temperatures were up to 50°C for *C. vulgare*; Fig. S3H). These microclimatic conditions might have been exacerbated by cutting the standing vegetation, which significantly reduced shade cover. In the growing season of 2022 (March to August), the total precipitation in the experimental sites was up to 50 % lower compared with the 30-year-average (Fig. S1). In addition, maximum temperatures reached 39.6 °C in July 2022, being on average 7°C above the maximum temperature of the previous 30 years (Fig. S1). The descendants of *M. ciliata* survived these dry and hot conditions at a higher rate compared to their ancestors, suggesting the evolution of higher drought tolerance or improved avoidance strategies. Drought avoidance strategies can involve increased root growth, xeromorphic leaf structures such as smaller and thicker leaves, higher stomatal density (Fang and Xiong, 2015) whereas drought tolerance involves improved osmoregulation (Morgan, 1984).

We found higher plant size and more leaves in descendants of *M. ciliata,* which may indicate greater acquisition and retention of resources as well as higher fitness. The higher performance of descendants can be explained by evolution of adaptation to the current environmental conditions over time (Pluess, 2013). In the case of *L. hispidus,* the initial size (measured in October 2021) of the descendants upon transplantation was much higher compared to the ancestors, but we could not detect any differences in the following spring. Thus, the descendants of *L. hispidus* performed better under controlled and comparatively unnatural conditions in the greenhouse, whereas they grew at a slower rate under field conditions and produced less leaves than the ancestors. We can see a similar tendency for *C. vulgare* regarding the interaction of origin with time of measurement for plant size. Here, descendants tended to grow slower than ancestors, which was also previously found in the same populations in a greenhouse study (Rauschkolb et al. 2022a). Reduced growth can be explained as a response to dry conditions as it reduces water loss by transpiration and reduces resource needs, and this explanation is supported by a multi-species experiment investigating ten grassland species under variable water availability treatments demonstrating that smaller seedlings have a higher survival rate (Harrison and LaForgia, 2019).

Despite slower growth of descendants compared to ancestors in *C. vulgare* and *L. hispidus*, we could not detect differences in mortality between temporal origins, which may be explained by the exceptional weather conditions during the growing season, since mortality reached 100% in August 2022 for both temporal origins and species. We also observed that larger plants tended to survive better overall, contradicting the notion that small plants are better adapted to dry conditions (Olson et al., 2018). We therefore conclude that the observed reduced plant size in descendants compared to ancestors seems to be a stress response rather than an adaptation to reduce evapotranspiration.

Overall, our findings indicate genetic differentiation between ancestors and descendants of our study populations in their natural habitat. Since the transplantation sites are very close to the collection sites (15 – 295m) and since the soil conditions are similar (Table S4), we can discuss the results with respect to evolutionary adaptation. Since we investigated rather isolated populations, we can attribute trait differentiation to evolutionary change by natural selection on standing variation rather than by immigration (Pluess, 2013). However, the population size is rather small for all three populations and neutral genetic drift may have played a role in trait differentiation. Moreover, the results for *M. ciliata* have to be interpreted with care, since we did not successfully grow a refresher generation to reduce storage and maternal effects (Franks et al., 2018). Another potential confounding factor can be the so-called "invisible fraction", *i.e.*, only a fraction of the seeds may have survived the storage conditions, which might be genetically correlated with post-emergence plant traits (Weis, 2018). However, since the germination rate of stored seeds was high in *M. ciliata* (100%) and *L. hispidus* (93%) and intermediate in *C. vulgare* (45 %), the invisible fraction cannot have influenced the results of *M. ciliata* and *L. hispidus* and the influence on *C. vulgare* might be small as well, given that stored seeds had a similar germination rate as newly sampled seeds (40%).

4.2. Timing and strength of selection pressure

The mortality data, assessed at several points in time, allowed us to investigate the strength of the selection pressure during different time periods. The highest selection during autumn-winter (October – April) was experienced by *C. vulgare* (37.5 - 42.3 % mortality, Fig. 3D) followed by *L. hispidus* (11.1 - 16.0 % mortality, Fig. 3G) and the lowest selection was found in for *M. ciliata* (0 - 13.3 % mortality, Fig. 3A). All three species are hemicryptophytes with renewal buds at ground level during winter (Niklas, 2008). The main causes of mortality in winter are usually freezing temperatures (Stushnoff et al., 1984). *Clinopodium vulgare* might be more exposed to the harsh weather compared to the other species, considering that the plants were transplanted on a slope from which the potentially protective vegetation layer was removed. Given the generally high mortality of all plants across species, any evolutionary changes over the last 23-26 years that would have conferred improved drought tolerance were not sufficiently strong to cope with the extreme conditions experienced in the study year. However, this observation applies only to juvenile plants as their root system is smaller than that of adult plants. In contrast, adult plants may have a deeper root system enabling them to survive under these conditions (Basu et al., 2016) and selection for improved drought tolerance at the adult stage may have been comparatively more successful.

Climatic predictions forecast more frequent extreme heatwaves and droughts in the next years (Dore, 2005; Ruosteenoja et al., 2018), which may further threaten the establishment and persistence of plant populations. This should be especially true if extreme conditions occur in consecutive years, since natural population dynamics becomes disturbed due to lack of recruitment and populations would have difficulties to recover from this (Crawley et al., 1990). In the case of *M. ciliata*, the strength of selection pressure depended on the temporal origin. The highest mortality increases for ancestors occurred between April and July 2022 (41.7 percentage points), and for descendants between July and August 2022 (36.7 percentage points), indicating that ancestors experienced selection pressure much earlier than descendants. These results support the idea that the population has evolved local adaptation to dryer conditions over the last 26 years leading to lower mortality.

4.3. Extending the resurrection approach by including *in situ* transplantations

The resurrection approach is a powerful method to study contemporary evolution of plant populations (Franks et al., 2018). It is constantly developed and applied in new contexts. Previous studies focused mainly on how single fast-growing annual species responded to experimental drought under controlled conditions (Hamann et al., 2021; Kooyers et al., 2021; Lambrecht et al., 2020). Recent resurrection studies also used multiple species and included treatments (*e.g.* herbivory, Rauschkolb et al., 2022b). However, these studies cannot prove whether the observed evolutionary changes are adaptive to their habitat of origin. With our current study, we propose and demonstrate in practice a novel methodological approach applying *in situ* transplantations within a resurrection ecology framework. Transplanting ancestors and descendants to the exact same location where they have been previously sampled provides researchers the ability to observe evolutionary change in the environment where the responsible selection pressures acted. In addition, seeds of ancestors and descendants can be sown at the collection sites in order to investigate evolutionary change in germination rate and seedling establishment. In our experimental demonstration, we found evidence of rapid adaptation in the study species. Nevertheless, we should consider the nursing period of the plants in the greenhouse prior to transplantation, which may prime ancestors and descendants differently.

We see strong potential in combining the resurrection approach with *in situ* transplantations and have further recommendations. First of all, we recommend monitoring the plants at the study sites over multiple years, which is important for perennials, especially if species need several years to flower, to gain a better insight into lifetime fitness. This can also enhance the robustness of the results by revealing natural fluctuation as a result of phenotypic plastic responses due to year-to-year variation in environmental conditions. Either the average phenotype across multiple years or the phenotypic expression during specific years may elicit plastic responses that reflect evolutionary adaptations. Secondly, we recommend the use of more than two generations, ideally a series of generations, to investigate the natural fluctuation in phenotypic expression across generations. This is important because it is often assumed that evolutionary changes build up gradually over time whereas these could also be the result of extreme selection events in single years (Gould and Eldredge, 1977). Thirdly, combining multi-year monitoring with continuous data logging of climatic variables may allow inference of the strength of selection induced by the environmental conditions over the different years. Finally, we suggest to include multiple populations of the same species, if these are available, to allow more general statements about the evolutionary potential of the species.

5. Conclusion

We investigated three perennial plant species using a novel approach that combined a resurrection experiment with *in situ* transplantations. We found evidence for evolutionary adaptations after 23 – 26 years of climate change in all species, but changes were most prominent in *M. ciliata.* Here, we observed lower mortality and larger plant size of descendants in their natural environment, suggesting evolution of local adaptation. In *C. vulgare* and *L. hispidus*, we found evolution of faster germination. In contrast, we found less growth in terms of plant size in the field conditions of the descendants from *C. vulgare* and lower production of leaves of descendants from *L. hispidus* compared to their ancestors. This indicates evolutionary differences, which did, however, not lead to lower mortality during that year's extreme drought period. Furthermore, mortality, a clear indicator of selection pressure, was highest in summer, where temperatures and drought peaked. These findings further support the notion that drought has been a key selective force for evolution in recent years and that adaptations to prolonged drought periods will play an important role for persistence of plant populations in the future (Franks et al., 2007; Nevo et al., 2012; Sekor & Franks, 2018; Thomann et al., 2015; Vigouroux et al., 2011). Evolutionary adaptations to drought include escape, avoidance and tolerance strategies (Basu et al., 2016), but also the evolution of seed traits such as dormancy and faster germination can be beneficial (Volaire and Norton, 2006).

Our study demonstrates that the incorporation of *in situ* transplantations into the resurrection approach is an important step to infer evolutionary adaptation. In future resurrection studies, the combination with *in situ* transplantations will be needed to confirm whether observed strategy shifts in common garden experiments translate to improved plant performance in their natural environments.

6. Appendix

6.1. Author contribution

PK, SG and JFS designed the study. SG and RR provided the seed material. PK, SG and JFS conducted the experiment. PK, MMS and JFS analyzed the data. PK wrote the manuscript with input from all co-authors.

6.2. Acknowledgements

We thank the Department of Nature and Forests (Public Service of Wallonia) for providing seed collection permits and for having authorized the experimental planting of *M. ciliata*. We thank the Natagora management commissions for validating the plantation and sowing of *L. hispidus* and *C. vulgare*. We are also grateful to Louis-Marie Delescaille, Alain Bouchat and Jean-Louis Gathoye for having identified the most suitable areas for installing the experimental plots taking into account the constraints related to the management of the nature reserves. We thank Susanne Pietsch, Robert Anton and all gardeners from the “Wissenschaftsgarten” of the Goethe University Frankfurt for their help in cultivating the seedlings and Yannick Coeckelberghs, Marie Karrenbauer, Emma Corvers, Isabel Aguirre Alcolea, Vera Holland and Romane Tardy for their substantial help in setting up and monitoring the experimental sites, and Andre Velescu for analyzing the soil samples. Finally, we thank the Royal Meteorological Institutes of Belgium and the Netherlands for providing the climate data.

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6.4. Data archiving

The data associated with this manuscript will be uploaded to a repository upon acceptance.

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Tables and Figures

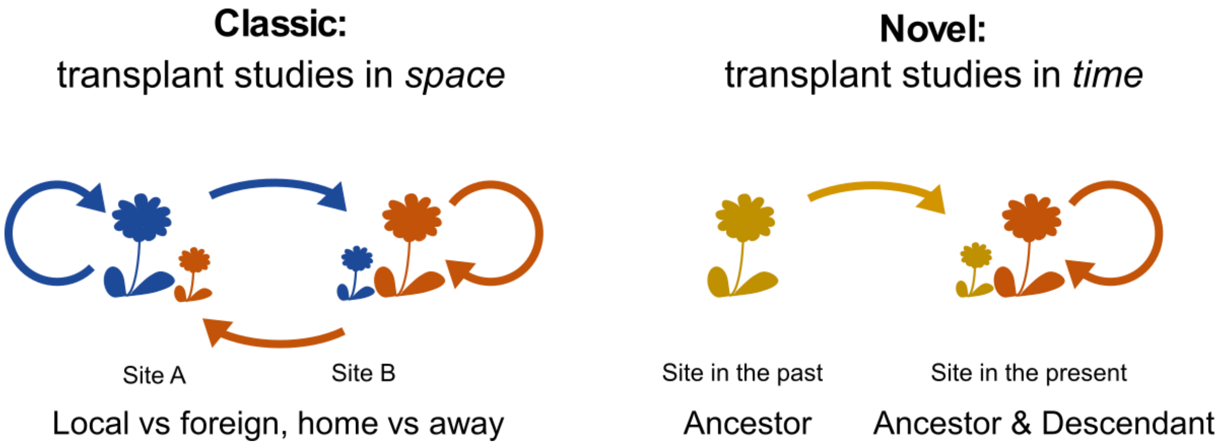

Figure 1. Comparison of the classical approach of transplant experiments in space (left) with the novel approach of transplant experiments in time (right). Classical reciprocal transplantation experiments aim to measure local adaptation and fitness trade-offs by transplanting plants from different populations reciprocally to all respective sites of origin. This method can conceptually be combined with the resurrection approach by transplanting ancestors and descendants of the same population to their collection site today. By doing so, comparisons of ancestors and descendants in their present-day natural environment can give insights into the evolution of local adaptation and underlying processes through time.

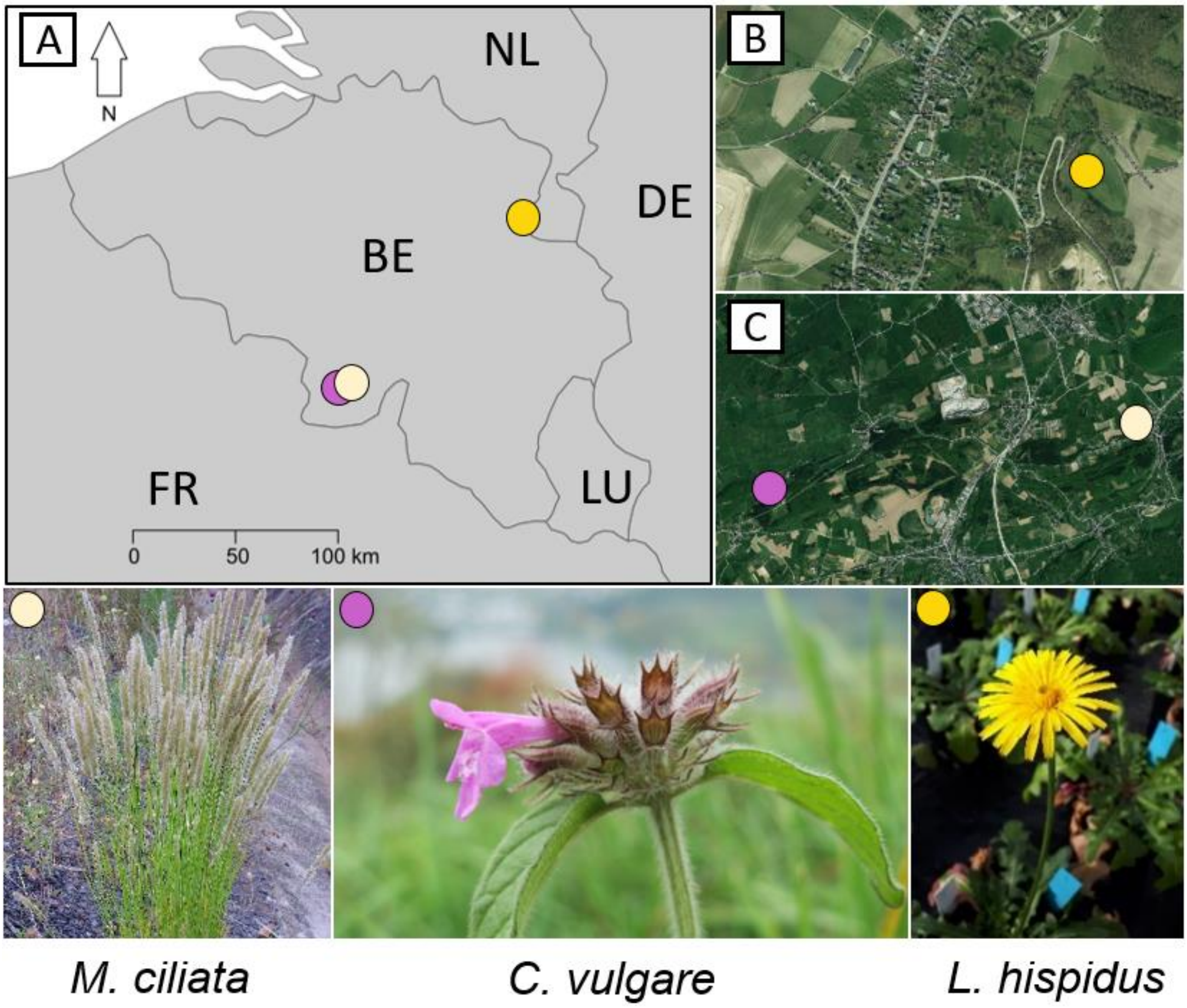

Figure 2. Map of the focal populations in nature reserves in Belgium. Overview map of Belgium (A) and zoomed-in areas of the focal populations (B & C). The populations of the species *M. ciliata* (grey circle), *C. vulgare* (pink circle) and *L. hispidus* (yellow circle) are located in Belgian nature reserves. Map A was made in R (version 4.0.3, R Development Core Team 2022) using the packages *map* and *mapdata*. Maps B and C were made in QGIS (QGIS Development Team (2023) using the plugin *maptiler*. The photos of *M. ciliata* and *C. vulgare* are in the public domain.

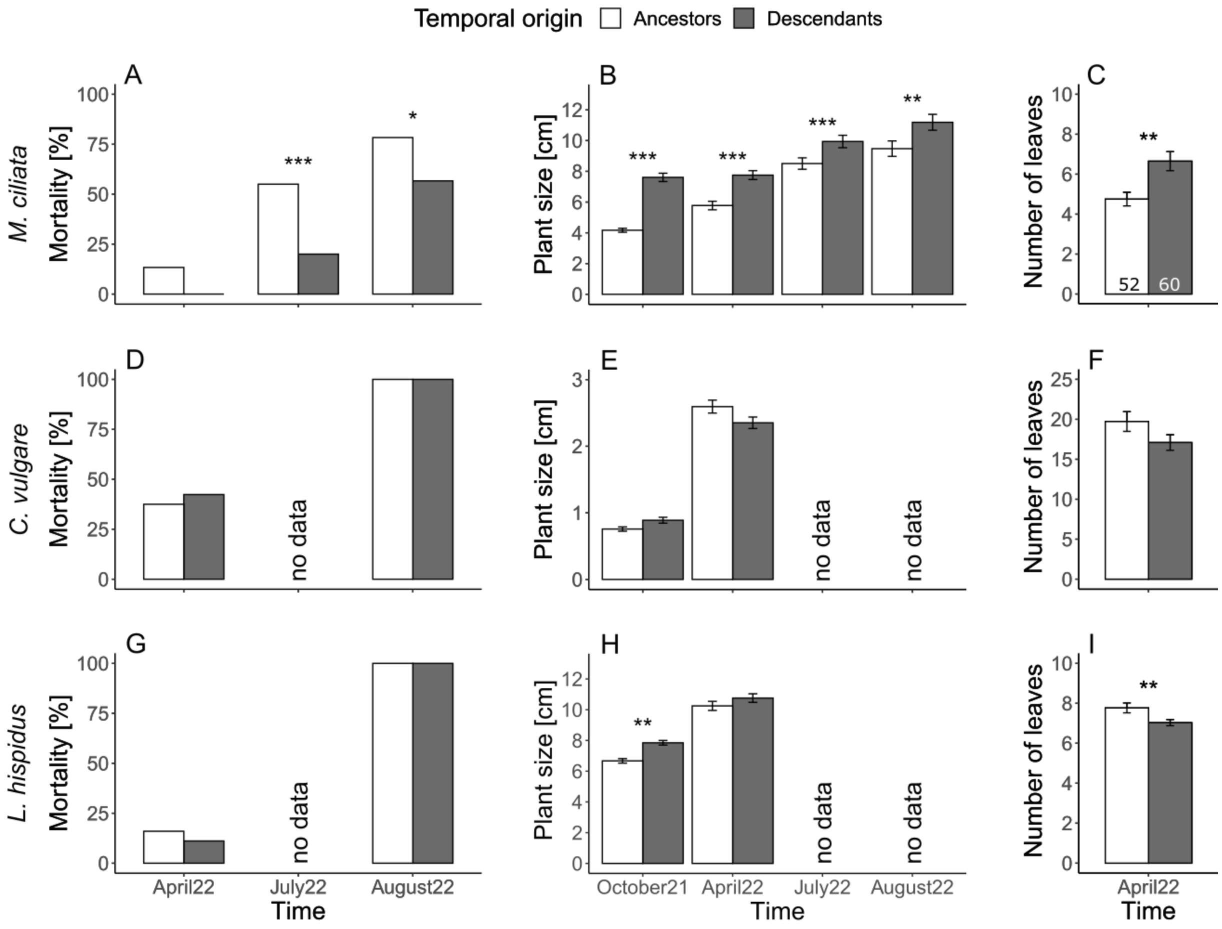

Figure 3. Mortality rates, plant size and number of leaves of *Melica ciliata* (A, B, C), *Clinopodium vulgare* (D, E, F) and *Leontodon hispidus* (G, H, I) in ancestors (white bars) and descendants (grey bars) measured at different time points. Shown are means and standard errors. Significant differences between ancestors and descendants in each point in time are indicated with asterisks (p = 0.05 – 0.01 *; p = 0.01 – 0.001 **; p < 0.001 ***). The sample sizes are indicated in the bars.

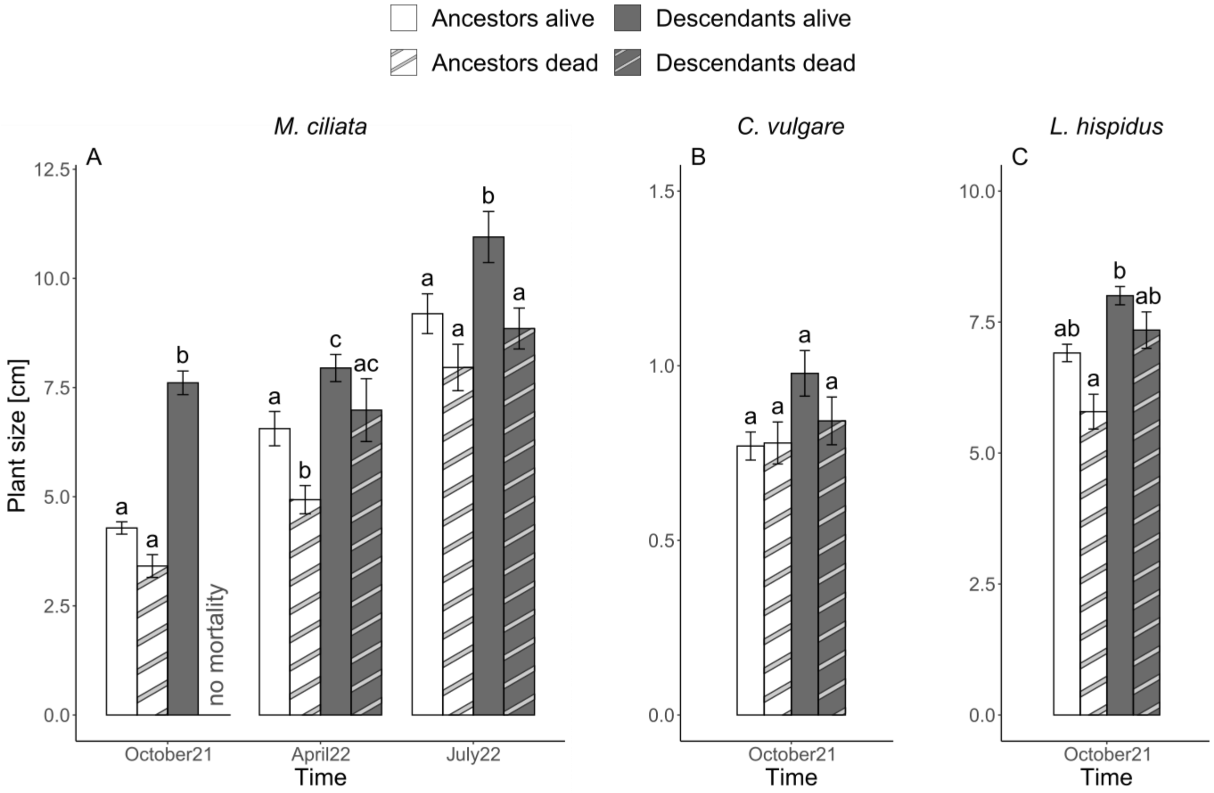

Figure 4. Plant size of *Melica ciliata* (A), *Clinopodium vulgare* (B) and *Leontodon hispidus* (C) grouped by their mortality status at the next measurement (dead indicated with stripes and alive indicated without stripes) of ancestors (white bars) and descendants (grey bars) at three different time points. Shown are means and standard errors. Significant differences in plant size between groups are shown with different letters within each time of measurement ($p < 0.05$). Note that we recorded 100 % mortality in *L. hispidus* and *C. vulgare* in August 2022, hence only the time period October 2021 – April 2022 could be analyzed.

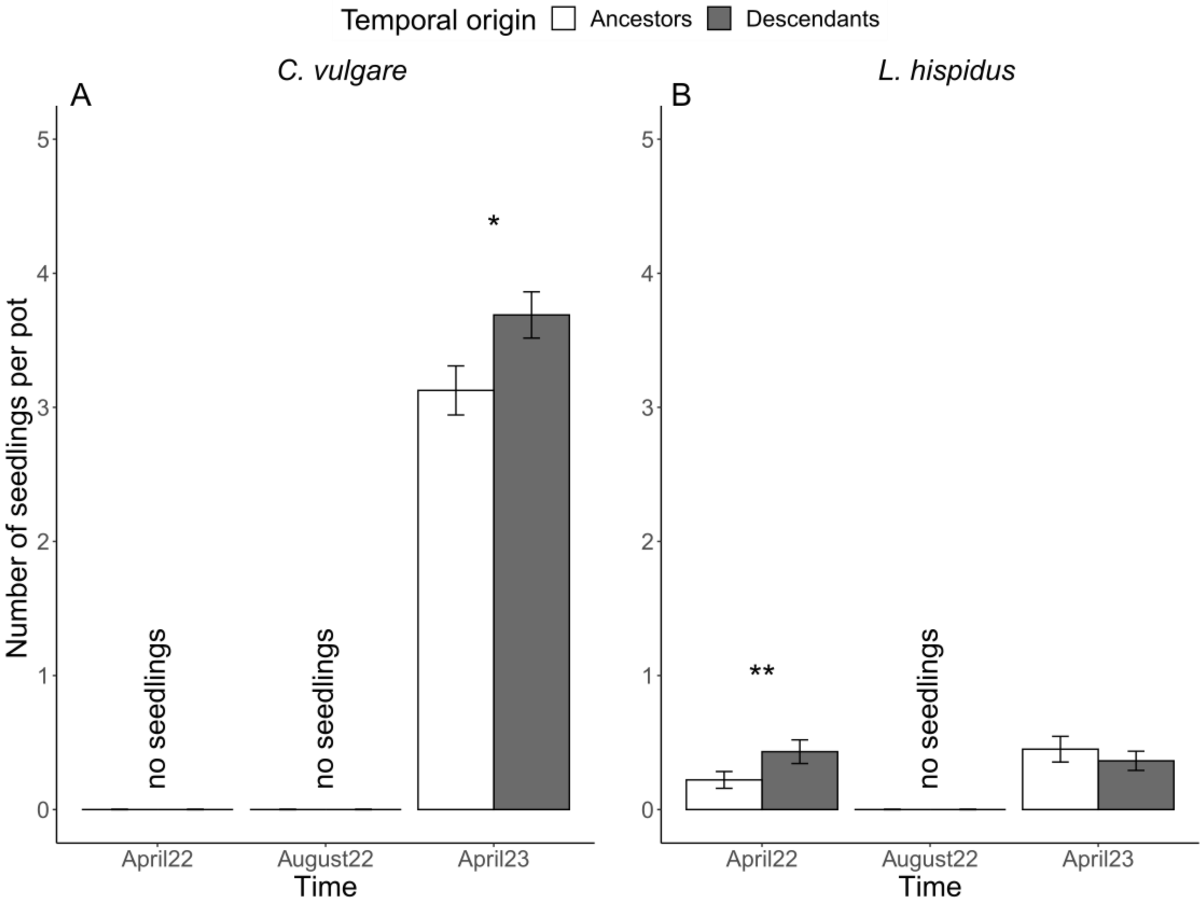

Figure 5. Number of seedlings germinated per pot from *C. vulgare* (A) and *L. hispidus* (B) in ancestors (white bars) and descendants (grey bars) measured at different time points. Shown are means and standard errors. Significant differences between ancestors and descendants are indicated with asterisks (p = 0.05 – 0.01 *; p = 0.01 – 0.001 **).

Table 1. Results of the statistical models testing the effects of temporal origin (ancestors, descendants), time of measurement (if measured more than once) and their interaction on mortality (GLMM), plant size (LMM) and number of leaves (LMM) of *Melica ciliata*, *Clinopodium vulgare* and *Leontodon hispidus*. For plant size, an additional LMM was created with temporal origin, time of measurement, mortality and their two- and three-way interactions. Shown are F-values and p-values for LMMs and Chi^2 and p-values for GLMMs. Significant p-values (< 0.05) are shown in bold.

Response variable	Explanatory variable	*M. ciliata*		*C. vulgare*		*L. hispidus*	
		Chi^2	p	Chi^2	p	Chi^2	p
Survival	Origin	20.73	<**0.001**	0.91	0.339	1.00	0.316
	Time	58.90	<**0.001**				
	Origin × Time	0.99	0.804				
		F	p	F	p	F	p
Plant size	Origin	51.36	<**0.001**	0.05	0.820	7.32	**0.013**
	Time	82.31	<**0.001**	706.17	<**0.001**	272.03	<**0.001**
	Origin × Time	6.63	<**0.001**	7.83	**0.006**	4.47	**0.035**
Leaf number	Origin	9.63	**0.002**	1.86	0.185	11.80	**0.002**
Plant size	Origin	45.13	<**0.001**	2.29	0.144	8.91	**0.005**
	Time	65.23	<**0.001**				
	Survival	13.29	<**0.001**	2.86	0.092	3.63	0.058
	Origin × Time	6.36	**0.002**				
	Origin × Survival	0.01	0.919	0.87	0.351	0.71	0.399
	Time × Survival	0.01	0.986				
	Origin × Time × Survival	3.24	0.073				

Table 2. Results of the statistical models testing the effects of temporal origin (ancestors, descendants), time and their interaction on number of seedlings after sowing (GLMM) of *Clinopodium vulgare* and *Leontodon hispidus*. Shown are Chi^2 and p-values. Significant p-values (< 0.05) are shown in bold. For *C. vulgare*, we only recorded seedlings at one time point (April 2023), hence we tested only the effect of temporal origin.

Response variable	Explanatory variable	*C. vulgare*		*L. hispidus*	
		Chi^2	p	Chi^2	p
Number of seedlings	Origin	4.719	**0.030**	1.103	0.294
	Time			1.935	0.380
	Origin × Time			8.666	**0.013**