Evolution of competitive ability and the response to nutrient availability: a resurrection study with the calcareous grassland herb, *Leontodon hispidus*

Pascal Karitter[1*], Emma Corvers[1], Marie Karrenbauer[1], Martí March-Salas[1], Bojana Stojanova[2], Andreas Ensslin[3], Robert Rauschkolb[4,5], Sandrine Godefroid[6], J.F. Scheepens[1]

[1]Plant Evolutionary Ecology, Institute of Ecology, Evolution and Diversity, Faculty of Biological Sciences, Goethe University Frankfurt, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany

[2] Department of Biology and Ecology, Faculty of Science, University of Ostrava, Chittussiho 10, CZ-710 00 Slezská Ostrava, Czech Republic

[3]Conservatory and Botanic Garden of the City of Geneva, Chemin de l´Impératrice 16 1, 1296 Chambésy, Geneva, Switzerland

[4]Institute of Ecology and Evolution with Herbarium Haussknecht and Botanical Garden, Department of Plant Biodiversity, Friedrich Schiller University Jena, Germany

[5]German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Leipzig, Germany

[6]Meise Botanic Garden, Nieuwelaan 38, 1860 Meise, Belgium

[*] Corresponding author: Pascal Karitter (karitter@bio.uni-frankfurt.de; +4915175074964)

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Conflict of interest

The authors have no conflict of interest to declare.

Author contribution

PK and JFS designed the study. SG and RR provided the seed material. EC, MK and PK conducted the experiment. PK, EC, MK, MMS and JFS analysed the data. PK wrote the manuscript and all authors helped to improve it.

Statement of inclusion

The authors of our study live in four different countries and include scientists who are based in the country where the seed material was collected and in the country where the study was conducted. Relevant literature published by scientists from the region where the seeds were collected was cited, and publications in the local language were considered. All authors were engaged with the research to ensure that the diverse sets of perspectives they represent were considered in the manuscript.

Data archiving

The data that support the findings of this study are available from Dryad [DOI to be inserted here after acceptance].

Given the strong degradation of natural habitats by nutrient enrichment, and the resulting increase in competition, understanding the ability of plant populations to adapt to changing conditions is highly important. Here, We contribute by investigated whether acommon calcareous grassland herb recently evolved its competitive ability and response to nutrient availability

Abstract

1. Rapid environmental changes across Europe include warmer and increasingly variable temperatures, changes in soil nutrient availability and pollinator decline. These abiotic and biotic changes can affect natural plant populations by imposing significant selection pressures and forcing plants to optimize resource use against competitors. Although recent studies have demonstrated the rapid and ongoing evolution of European plants to global change dynamics, the evolution of competitive ability in the context of changes in nutrient availability remains understudied.
2. In this study, we investigated whether the common calcareous grassland herb *Leontodon hispidus* recently evolved its competitive ability and response to nutrient availability. We grew ancestors sampled in 1995 and descendants sampled in 2018 from a single population under common conditions and applied a competition treatment using the natural competitor *Brachypodium pinnatum*. Furthermore, we applied nutrient treatments to plants grown under competition, supplying plants weekly with either no fertilizer, or with nitrogen, phosphorus, or both.
3. We found evidence for evolution of increased competitive ability, with descendants producing more vegetative biomass than ancestors when grown under competition. The competitive ability also depended on the nutrient treatment, indicating that descendants might be adapted to lower nitrogen concentrations, which could be linked to the decreasing nitrogen emissions into the atmosphere since the 1990s. Furthermore, we observed evolution of taller flower stems, which may reflect a strategy to increase pollinator visits under the existing pollinator decline in recent decades.
4. Overall, our study demonstrates rapid contemporary evolution, but also the complexity of the underlying processes of contemporary evolution, and sheds light on the importance of understudied potential selection agents such as nutrient availability.

Keywords: Competition; Contemporary evolution; Fertilization experiment; Global change; Rapid evolution; Resurrection approach

Introduction

Environmental conditions have been rapidly changing for decades and are affecting ecosystems worldwide (IPCC, 2018). These rapid changes include, among others, higher frequencies and intensities of droughts and heatwaves (Dore, 2005; Ruosteenoja et al., 2018; Samaniego et al., 2018), pollinator decline (Potts et al., 2010), and changes in nutrient availability (Galloway et al., 2008; Newman, 1995; Smith et al., 1999). These abiotic and biotic changes can disturb natural plant populations by imposing significant selection pressures and forcing plants to optimize their resource use against competitors (Bonser & Ladd, 2011; Gao et al., 2022; Mosquin, 1971).

Agricultural land use and fossil fuel combustion contribute to the continuous release of nitrogen (N) and phosphorus (P) into ecosystems worldwide through extensive fertilization and deposition from the atmosphere (Galloway et al., 2008; Newman, 1995; Smith et al., 1999). Excess agricultural fertilizer can be released to adjacent ecosystems via runoff or transport by freshwater bodies (Ceulemans et al., 2014). Since the beginning of the industrial revolution, the yearly release of N in the biosphere increased from 15.3 to 259 Mt and of P from 0.3 to 16 Mt (Peñuelas et al., 2012). Whereas N emissions have been steadily decreasing again since the 1990s (European Environment Agency, 2021), P levels are still above the recommended ranges in many agricultural soils in Europe (BDB, 2005; Djodjic et al., 2004; Ketterings et al., 2005; Reijneveld et al., 2010). Phosphorus has a much slower amelioration over time than N and thus, the effects of P enrichment are also likely to be more persistent in the future (Parkhurst et al., 2022). These shifts in the availability of N and P are likely affecting plant populations, and rapid adaptation to those changes will be essential for population persistence (Sala et al., 2000; Tilman et al., 2001). While the impact of an excess of N on plants has been widely studied since decades now, especially through atmospheric N deposition (Bobbink et al., 1998; Clark & Tilman, 2008; Cleland & Harpole, 2010; Conley et al., 2009; Phoenix et al., 2006; Stevens et al., 2004), the effect of P enrichment has received less attention (but see Ceulemans et al., 2011, 2014; Janssens et al., 1998; van Dobben et al., 2017). The effects of nutrient enrichment can have big impacts on plant populations through competitive exclusion, higher susceptibility to pests and abiotic stressors, soil acidification, and even through toxicity (Bobbink et al., 2010; Hautier et al., 2009; Johnson, 1993; Olsson & Tyler, 2004; Stevens et al., 2010).

Since plants are continuously competing for space and resources such as light, water and nutrients (Craine & Dybzinski, 2013), changes in the availability of these resources will strongly affect the evolution of plant responses since less competitive species are likely to experience higher mortality (Grime, 1973). An increase in soil nutrient resources causes increased aboveground vegetative growth in general, but will also increase shading and thereby reduce light availability for smaller plants. Competition for above- and belowground resources therefore changes with plant productivity (Rajaniemi, 2002). In originally nutrient-poor habitats, competition may shift from below- to aboveground when nutrients suddenly become abundant (Hautier et al., 2009), while a reduction in nutrients causes stronger belowground competition (Newman, 1973). Here, plants may increase their root length to acquire more nutrients for themselves, while at the same time this reduces the nutrient availability for their competitors (i.e., supply pre-emption, Craine et al., 2005). Nutrient availability can also be affected by the climate-change related increase in the occurrence of droughts. This is because root uptake of most mineral nutrients depends on soil moisture (Taiz & Zeiger, 2006). Additionally, the enzymatic activity of soil microorganisms may also be affected by droughts, leading to impairment of nutrient mineralization (Silva et al., 2010). Hence, depending on the soil physiochemical properties nutrient availability can be low for plants, even if nutrient concentrations are high (Amtmann & Blatt, 2009). Consequently, changes in nutrient supply and resulting impacts on competition can impose strong selection pressure on plants to evolutionarily increase their competitive ability through adjusting growth-related traits under the novel environmental conditions (Craine & Dybzinski, 2013; Falster & Westoby, 2003). Given the strong degradation of natural habitats by nutrient enrichment and the resulting increase in competition, understanding the ability of plant populations to adapt to these changing conditions is of high importance (Ceulemans et al., 2013, 2014; Hautier et al., 2009; Smith et al., 1999; Stevens et al., 2010).

Over the recent decades, the resurrection approach has been widely used to study rapid evolution of plant populations (Franks et al., 2018; Hamann et al., 2021; Rauschkolb et al., 2023; Thomann et al., 2015; Wooliver et al., 2020). This approach involves an experimental design that utilizes seeds collected from a population before (ancestors) and after (descendants) a potential selection pressure, such as consecutive drought years. Comparisons of the phenotypes of these two generations in a controlled environment can then uncover evolutionary changes (Franks et al., 2007). Resurrection studies have provided compelling evidence that plant populations can undergo rapid evolution in various morphological, physiological, and phenological traits within just a few generations (Franks et al., 2007; Hamann et al., 2018; Nevo et al., 2012; Sekor & Franks, 2018; Thomann et al., 2015; Thompson et al., 2013). Whereas the evolution of competitive ability has been studied in some resurrection experiments (Frachon et al., 2017; Sultan et al., 2013; Ziska, 2017), resurrection studies on evolutionary responses to nutrient availability are currently, to our knowledge, lacking. Sultan and colleagues (2013) conducted a resurrection study on the invasive species *Polygonum cespitosum* and found evolution of higher competitive ability after 11 years through higher reproductive output, and stronger plasticity in physiological traits and root allocation. Frachon and colleagues (2017) found that *Arabidopsis thaliana* responded to local warming and increased competition through a delay in bolting time and evolution of an adaptive strategy that mainly involved the tendency to escape competition in crowded environments through lateral growth. Since competitive ability is also highly dependent on abiotic factors, it is important to examine evolution of competitive ability in the context of changing nutrient availability to gain a deeper understanding of plant responses to environmental changes.

Here, we conducted a resurrection study to investigate recent adaptive evolution of *Leontodon hispidus* (Asteraceae), a common herb in calcareous grasslands, to N and P enrichment and competition. We used ancestors sampled in 1995 and descendants sampled in 2018 (i.e., a 23-year difference) of one population in a Belgian nature reserve. After two refresher generations, we grew ancestors and descendants under common conditions and applied a competition treatment using the natural competitor *Brachypodium pinnatum* (Poaceae). Furthermore, we applied nutrient treatments to plants that were subject to competition, supplying those plants weekly with either no nutrients, or with nitrogen, phosphorous, or both. We measured growth, leaf and floral traits. We hypothesized that the decrease in soil nutrient availability lead to a shift from aboveground competition for light to belowground competition for nutrients. Thus, we expect evolution of lower competitive ability aboveground and higher competitive ability belowground. Further, we hypothesized that the decrease of N emissions over the last decades selected for higher fitness in descendants of *L. hispidus* under low N availability. In contrast, we expect that descendants and ancestors respond similarly to high P availability due to slower reduction of P emissions and greater persistence in the soils in the last decades.

Material and methods

Study species and seed origin

Leontodon hispidus L. (Asteraceae) is a perennial rosette-forming herbaceous plant. It is self-incompatible and can flower in the first year after germination, which typically occurs from June to October (Kühn & Klotz, 2002). It is widespread throughout Europe and commonly found in calcareous grasslands, which received conservation priority by the European commission ("Festuco-Brometalia"; EU code 6210: Semi-natural dry grasslands and scrubland facies on calcareous substrates). Calcareous grasslands are threatened by eutrophication and lack of management (Habel et al., 2013) and *L. hispidus* as a typical species for this habitat is steadily declining in the northern parts of Belgium (Hoste et al., 2006).

Seed material was collected from one population in a nature reserve called “Thier à la Tombe” in the northeastern part of Belgium (50°47'34.7"N, 5°40'22.6"E) in two temporal origins: 1995 (ancestors) and 2018 (descendants). The vegetation is a calcareous grassland that was unmanaged until 2007, after which sheep grazing was introduced yearly in spring and early summer. The nature reserve is situated on a west-facing slope next to an agricultural field. The distance to the nearest other population is approximately 2 km, decreasing the likelihood of cross-pollination between populations of *L. hispidus*. The ancestral seed collection was conducted by the Meise Botanic Garden (Belgium) for conservation purposes. Although the precise number of sampled individuals was not recorded, efforts were made to represent the genetic diversity of the population in the sampling. All seeds were cleaned, bulked, and dried at 15 % relative humidity. Finally, the seeds were stored at -20 °C in the seed bank of Meise Botanic Garden. In summer 2018, we revisited the population and collected the seeds from all inflorescences from 20 mother plants. These seeds were cleaned, bulked and then stored at 4 °C. To ensure that the ancestral seed material is comparable to descendant seed material, and that both samplings represent the genetic diversity of the population, Rauschkolb et al. (2022a) analyzed the genomic relatedness of both temporal origins. Analysis showed similar relatedness of plants in the seed material of ancestors and descendants, as well as similar allelic richness, altogether indicating that the genetic structure is comparable between samplings, that sufficient seed material was collected, and that there is low influence of bottlenecks or gene flow (Rauschkolb et al., 2022a).

Experimental design

Both ancestral and descendant seeds were grown for a refresher generation (Rauschkolb, et al., 2022b) in order to reduce environmental, maternal and storage effects (Franks et al., 2018). We sowed 300 seeds from each temporal origin and selected 15 random individuals for each temporal origin that were haphazardly pollinated by hand in cages to prevent unintentional cross-pollination (Rauschkolb, et al., 2022b). Due to inadequate seed production from some seed families, we grew a second refresher generation using the seed material obtained from the first refresher generation. We cultivated the plants in the same conditions and we used bumblebees (Natupol seeds, Koppert GmbH, Straelen, Germany) as pollinators. Ultimately, nine seed families from both ancestral and descendant temporal origin yielded sufficient seed material for the experiment.

In March 2022, we prepared 25 pots (1.5 L) for each maternal line with nutrient-poor soil (Einheitserde Typ 1, Einheitserde, Sinntal-Altengronau, Germany) in the greenhouse and sowed 3 seeds into each pot. Simultaneously, we sowed 150 g of seeds of *Brachypodium pinnatum* (UG12, Rieger Hofmann GmbH, Blaufelden-Raboldshausen, Germany) in 6 trays using the same nutrient-poor soil. *Brachypodium pinnatum* was used as a competing grass in this experiment as it is a natural competitor of *L. hispidus* in its natural habitat. All pots and trays were watered three times a week to soil capacity, meaning that the soil could not take up any more water after each watering event. Once the *L. hispidus* seedlings emerged and all seedlings developed their first true leaf, we thinned them to a single individual per pot and moved this individual to the center of the pot. Three weeks after germination, we started the nutrient and competition treatments. To prevent nutrient deficiencies, we first added 1.2 grams of slow-release fertilizer (Osmocote Pro, Controlled Release Fertilizer 3-4, ICL Group, Ludwigshafen, Germany) to each pot.

We divided the pots into 5 treatment groups with 5 replicates per seed family and applied the following competition and nutrient treatments: (i) without competition and without fertilizer (i.e., without competition control); (ii) with competition and without fertilizer (with competition control); (iii) with competition and nitrogen fertilizer (N); (iv) with competition and phosphorus fertilizer (P); (v) with competition and nitrogen + phosphorus fertilizer (NP) (Fig. 1). In the competition groups, we transplanted four individuals of *B. pinnatum* with approximately 10 cm height into each pot with an equidistance of 5 cm around the center of the pot (Fig. 1). For the N source, we used urea (CH_4N_2O, Roth, Karlsruhe, Germany) and for the P source, we used monosodium phosphate (NaH_2PO_4, Roth, Karlsruhe, Germany). We chose these fertilizers, as they only contain the macronutrient of interest and no additional macronutrients (Marschner, 1995). The plants were watered three times per week to soil capacity and weekly with their respective fertilizer solution to simulate constant nutrient influx: 17.86 mg urea (≈ 10 mg N) in 20 ml H_2O for the N-treatment; 21.92 mg monosodium phosphate (≈ 5 mg P) in 20 ml H_2O for the P-treatment. These concentrations were chosen as they simulate a strong influx of nutrients which is comparable to the yearly influx of nutrients into ecosystems: 17 kg N/ha/year and up to 5 kg/P/ha/year (Newman, 1995; Stevens et al., 2004). Plants in the NP-treatment received the N- and P-treatment consecutively. In total, the experiment consisted of 450 pots (2 temporal origins × 9 seed families × 5 treatment groups × 5 replicates). We randomized all pots every two weeks and moved the pots to an outdoor common garden after four weeks.

Plant measurements

During the course of the experiment, we recorded the onset of flowering and the height of the first flower stem of *L. hispidus* every Monday, Wednesday and Friday. We defined flowering onset as the point when the first anther became visible. After 17 weeks, all plants had flowered and we harvested them after measuring the rosette diameter. We collected all the flower heads and stems as reproductive biomass and the leaves as vegetative biomass. For each individual, three randomly selected healthy and fully developed leaves were sampled and their combined area was measured with the smartphone application “easy leaf area free” (Easlon & Bloom, 2014). The leaves were dried in a drying oven at 60 °C for three days and then weighed at a high-precision scale (CPA225D-0CE, e = 1 mg, Sartorius AG, Göttingen, Germany). We calculated specific leaf area (SLA) by dividing the combined leaf area by its dry weight. The root biomass of *L. hispidus* was separated from the roots of the grasses and washed to remove soil. The root biomass, vegetative biomass and reproductive biomass were separately dried in a drying oven at 60 °C for 72 hours and then weighed at the high-precision scale as well. For the final values of vegetative biomass, we added the dry weight of the three leaves we collected for the leaf area measurements. Finally, we calculated reproductive investment as the ratio of reproductive biomass to vegetative biomass.

Soil analysis

In autumn 2021 we took soil samples of 25 cm^3 at 10 cm soil depth at four random locations in the natural population of *L. hispidus*. All four soil samples were bulked and dried at 40 °C for one week in a drying oven. We sieved the samples to < 2 mm, and we milled 0.3–1 g of the sieved soil with a Mixer Mill MM400 (Retsch, Haan, Germany) for 60 seconds with 30 rounds per second. To avoid contamination between samples, we cleaned the sieving and milling tools between samples with an air-compressor and water. The samples were then analyzed to determine the amount of fundamental minerals (total element content of P, K, S, Ca, C, N and

S), as well as pH level and salinity (Table S1). Total C and N measurements were performed by elemental analysis through thermal combustion and thermal conductivity detection of CO_2/N_2 (Thermo Scientific, Flash 2000 HT Plus, Bremen, Germany). For total element concentrations, we digested the samples with a mixture of HNO_3, HF and H_2O_2 (4:2:1) in a microwave oven (Mars 6, CEM, Kamp-Lintfort, Germany). Then we complexed excess HF with H_3BO_3 and measured total element concentrations by ICP-OES. We confirmed complete element recovery of total digestions with certified reference material (BCR2, Columbia river basalt).

Data analysis

Since we were specifically interested in the effects of competition *per se* and of the nutrient treatments *per se*, we divided and analysed the data in two subsets. To analyse the effect of competition on the temporal origins, we included only the groups without fertilizer (i.e., without competition control, and with competition control) in the first subset. The second subset contained all groups with nutrient treatments (N, P and NP) and the competition group without fertilizer (with competition control). All statistical analyses were performed using R (version 4.0.3, R Core Team, 2020). We performed linear mixed-effects models (LMMs) using the *lmer* function implemented in the *lme4* package (Bates et al., 2015) to analyse the following response variables: vegetative biomass, rosette diameter, root biomass, SLA, reproductive biomass, reproductive investment, flower stem height, and onset of flowering. Using the competition data set, we tested for effects of the competition treatment, temporal origin and their interaction as fixed factors and seed family nested in temporal origin as random factor. Using the nutrient treatment data set, we tested for effects of the nutrient treatment, temporal origin and their interaction as fixed factors and seed family nested in temporal origin as random factor. When the normality and homoscedasticity of model residuals were not met, we applied appropriate transformations to the response variables (see transformations in Table 1 and Table 2). All linear models were analysed using the *Anova* function (Type I) and analyses were always followed by Tukey post-hoc tests for each treatment pair within temporal origins and for each temporal origin within each treatment using the *emmeans* package (Lenth, 2021).

Results

According to the LMMs, the competition treatment had a significant effect on all measured traits except onset of flowering (Table 1), while significant differences between ancestors and descendants were found in vegetative biomass, rosette diameter, SLA, and flower stem height (Table 1). Competition had contrasting effects on ancestors and descendants in vegetative biomass and root biomass, as indicated by the significant interaction between competition and temporal origin (Table 1). Post-hoc comparisons show that without competition, descendants and ancestors did not differ in their vegetative biomass or root biomass, but competition led to lower vegetative and root biomass in ancestors compared to descendants (Fig. 2AC). Without competition, descendants had a significantly larger rosette diameter compared to ancestors (Fig. 2B) and taller flower stems (Fig. 2G), but did not differ in the remaining traits (Fig. 2). Competition generally decreased rosette diameter, reproductive biomass and the reproductive investment in both temporal origins, but these traits were not significantly different between ancestors and descendants (Fig. 2BEF). Onset of flowering was not significantly affected by competition nor differed between ancestors and descendants (Fig. 2H). Finally, competition also increased SLA for ancestors and descendants, but post-hoc tests show that descendants had a significantly lower SLA compared to ancestors under competition (Fig 2D).

The nutrient treatments significantly affected the rosette diameter (Table 2) and the onset of flowering (Table 2). Temporal origin affected rosette diameter, root biomass, SLA, flower stem height significantly, and vegetative biomass marginally significantly (Table 2), while a significant interaction between the nutrient treatments and temporal origin was found only for root biomass (Table 2). According to the post-hoc comparisons, the N-treatment showed increased rosette diameter of ancestors compared to the control (Fig. 3B), but the rosette diameter was not significantly affected by other nutrient treatments. In contrast, the N-treatment did not affect the rosette diameter of descendants, whereas the NP-treatment increased the rosette diameter of descendants compared to the control (Fig. 3B). The N-treatment decreased the root biomass and the reproductive biomass of descendants compared to the control (Fig. 3CE) leading to no significant differences of root biomass between descendants and ancestors in the N-treatment (Fig. 3C). Furthermore, descendants flowered later in the N-treatment compared to the P-treatment (Fig. 3H). According to the post-hoc comparisons, significant differences between ancestors and descendants in the control treatment, if any, disappeared in the N-treatment. In the P-treatment, descendants maintained higher root biomass (Fig. 3C), lower SLA (Fig. 3 D) and taller flower stems (Fig. 3G) compared to ancestors, but vegetative biomass and rosette diameter lost differences between ancestors and descendants. Finally, in the NP-treatment, descendants maintained their larger rosette diameter compared to ancestors (Fig. 3B).

Discussion

In order to study the evolution of competitive ability and of responses to changing nutrient availability over the last decades, we conducted a resurrection study using ancestors collected 30 years ago and descendants collected in 2018 after growing two refresher generations. We found evidence for evolution of higher competitive ability in descendants, as they showed better growth than ancestors when grown under competition. Furthermore, combining competition with nutrient treatments revealed that competitive ability also depended on the nutrient conditions.

Evolution of competitive ability

The competitive ability of plant populations and their evolution may be strongly affected by the highly diverse environmental changes over the last 25 years that include changes in climate (e.g., heatwaves and droughts), changes in nutrient availability, pollinator decline, and changes in grazing regime (Simon & Schmidt, 2017). The competition treatment in our experiment had a very strong effect on growth-related traits (e.g., vegetative and root biomass). We observed that the competitor *B. pinnatum* was growing much taller than the rosettes of *L. hispidus*, which were substantially shaded as a consequence. Hence, *L. hispidus* received less light and competed for nutrients and space. Even though both ancestors and descendants were strongly affected by the competition, descendants outperformed ancestors for most growth-related traits (higher vegetative and root biomass, larger rosette diameter, taller flower stems) and maintained lower SLA. Regarding competition for light and space, descendants had a larger rosette diameter, and thus were able to capture more light. Notably, the larger rosette diameter of descendants did not trade off with leaf thickness, as indicated by the lower SLA.

We expected that the evolution of higher belowground competitive ability would come at the expense of aboveground competitive ability due to a decrease in soil nutrient availability over the last decades. Accordingly, we found compelling evidence that this population of *L. hispidus* has evolved higher competitive ability through faster growth belowground, but also faster growth aboveground, making this species a stronger competitor for light and nutrients. Consequently, selection for competitive ability could either be facilitated directly by increased competition or indirectly by other selection agents that increase competitive ability as a side effect (e.g., low water availability selecting for faster root growth also makes plants more competitive belowground). It is possible that the environmental changes of the recent decades did not lead to a shift to belowground competition, but applied selection pressures both below- and aboveground equally. Faster growth is especially important to establish in the early life stage or early in the season, when interspecific shading is still minimal.

Furthermore, *L. hispidus* is highly dependent on pollinators for reproduction since it is a self-incompatible species (Kühn & Klotz, 2002). The pollinator decline during the recent decades might affect the selection pressure of plants aboveground as plants compete for pollinators (Potts et al., 2010). We found evolution of taller flower stems, which can be beneficial to better compete for pollinators by making the flowers more visible (Engel & Irwin, 2003) and even though we did not study pollinator decline as a direct agent of selection, the evolution of taller flower stems makes senses in the context of pollinator decline during the recent decades (Potts et al., 2010). Competition for pollinators can also result in evolution of selfing (Eckert et al., 2010; Thomann et al., 2013), but the breakdown of self-incompatibility is often a slow process (Cheptou & Avendaño, 2006; Lafuma & Maurice, 2007) and the self-incompatibility is very likely constraining evolution towards selfing in *L. hispidus*. In line with our findings, another resurrection study by Thomann and colleagues (2015) found evolution of larger flowers and flower longevity after 18 years in a population of the annual *Centaurea cyanus,* also a strongly self-incompatible species. Accordingly, it is possible that *L. hispidus* also evolved other floral traits such as capitula size, floral display, flower longevity or flowering duration, which should be considered in future studies.

Responses to nutrient enrichment

The soil analyses of the original population site revealed a N content of 0.49 %, which is comparable to other grasslands (Piqueray et al., 2011) and probably decreased in the studied site due to reduction of emissions since the 1990s (European Environment Agency, 2021; Klein et al., 2019). The total P content on the other hand was 530 mg/kg in our studied site (Table S1) and is much lower in comparison to other calcareous grasslands, which can reach over 1000 mg/kg of total P content (Alt et al., 2011; P. J. Wilson & Wheeler, 2016). A possible explanation for the lower P content in the original site of *L. hispidus* could be that the slope of the site is increasing the runoff of nutrients and thus, P is being washed out from the soil quickly and cannot accumulate in high quantities (Li et al., 2006).

While descendants generally outperformed ancestors without nutrient addition, adding nutrients generally reduced the differences between ancestors and descendants, which was most evident in the N- and NP-treatment, but less in the P-treatment. Adding N removed all significant differences between ancestors and descendants compared to the control. This suggests that descendants have evolved an increased ability to compete for N, since supplementing N no longer gives them an advantage due to decreased belowground competition (Newman, 1973; Wilson & Tilman, 1993). Nitrogen depositions decreased over the last three decades and descendants might thus have evolved adaptations to lower N availability. This is further evidenced by the descendants in the treatments with low N availability (control, P-treatment), where we observe higher belowground competitive ability (i.e., higher root biomass) of descendants compared to ancestors. Chronic addition of nutrients (especially N) has been shown to decrease N use efficiency (NUE) of plants and has strong links to plant evolutionary history (Egan et al., 2019; Liao et al., 2021). Hence, it is likely that ancestors of *L. hispidus* also had evolved a low NUE due to high N emissions, while the subsequent decrease in emissions likely favoured plants with higher NUE. It remains challenging to pinpoint the main underlying selective agent but nonetheless, our results indicate that competitive ability is very dependent on the nutrient availability and it is very likely that nutrients play a significant part in the evolution of competitive ability.

We found significant differences between ancestors and descendants in the P-treatment in several traits, such as larger root biomass or taller flower stems in descendants. However, these results did not differ from the control and therefore provide no evidence for evolution of P uptake strategies in the studied population. This finding is in line with the assumption that the availability of P did not significantly change in the recent decades and, as a consequence, did not act as a potential selection agent. We only used one fixed concentration for each nutrient treatment, whereas using multiple concentrations in an experiment would give more insight into underlying processes, since plant responses might vary greatly depending on concentrations. We also applied the nutrient treatments only to plants growing under competition due to space constraints meaning that we cannot disentangle the interaction of competition and nutrient availability. Conducting a resurrection study using a full factorial design with competition and nutrient treatments could give further insights into the relationships between competition and nutrients as well as their evolution. Furthermore, we only studied a single population of *L. hispidus*, making it difficult to generalize the results to the species level. Moreover, we had little data available on the local changes of relevant environmental factors that the ancestors and descendants experienced.

Conclusion

In this study, we found evidence for evolutionary changes in competitive ability and responses to changes in nutrient availability. Furthermore, supplementing nutrients (especially N) reduced differences in competitive ability between ancestors and descendants, suggesting that nutrients are a limiting factor in interspecific competition. We also found evolution of taller flower stems, which could be linked to pollinator decline as a means to increase the competitive ability for pollinator visits. Overall, the results of our study demonstrate the complexity of underlying processes of contemporary evolution and shed light on the importance of understudied potential selection agents that can be investigated using resurrection studies. Especially studying the effects of decreasing N emissions on plant populations after strong eutrophication will provide valuable insights for evolutionary responses of plant populations in the future.

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Tables and Figures

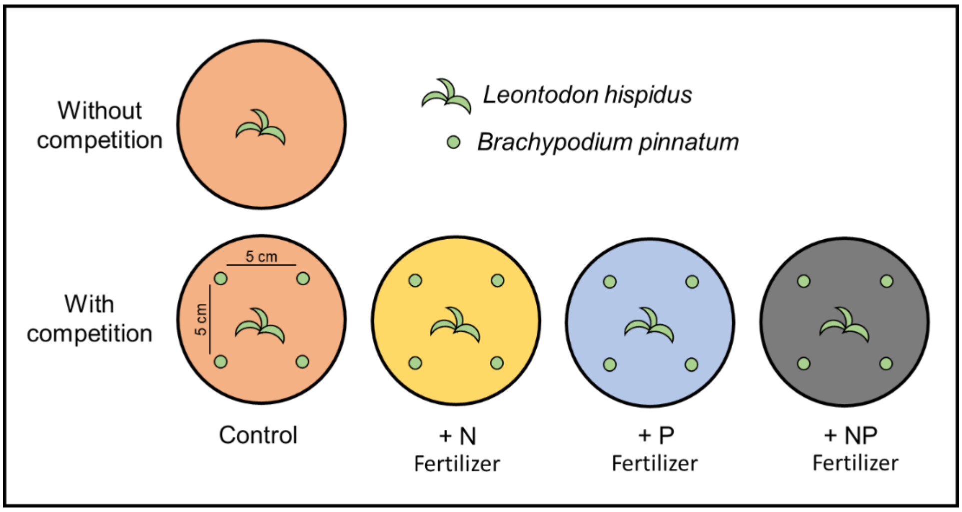

Figure 1. Experimental design of the study. Ancestors and descendants of *Leontodon hispidus* were cultivated in pots and divided into 5 treatment groups. One group was cultivated without competition and no additional nutrient supply. The other four groups were all grown with competition in combination with a weekly nutrient treatment (control, N fertilizer, P fertilizer, NP fertilizer). For the competition treatments, we used *Brachypodium pinnatum* which naturally occurs in the habitat of *L. hispidus* and is one of its strongest competitors. Each competition treatment involved the transplantation of four individuals of c. 10 cm tall *B. pinnatum* plants around *L. hispidus* in the centre with an equidistance of 5 cm once the *L. hispidus* plants developed their first true leaves.

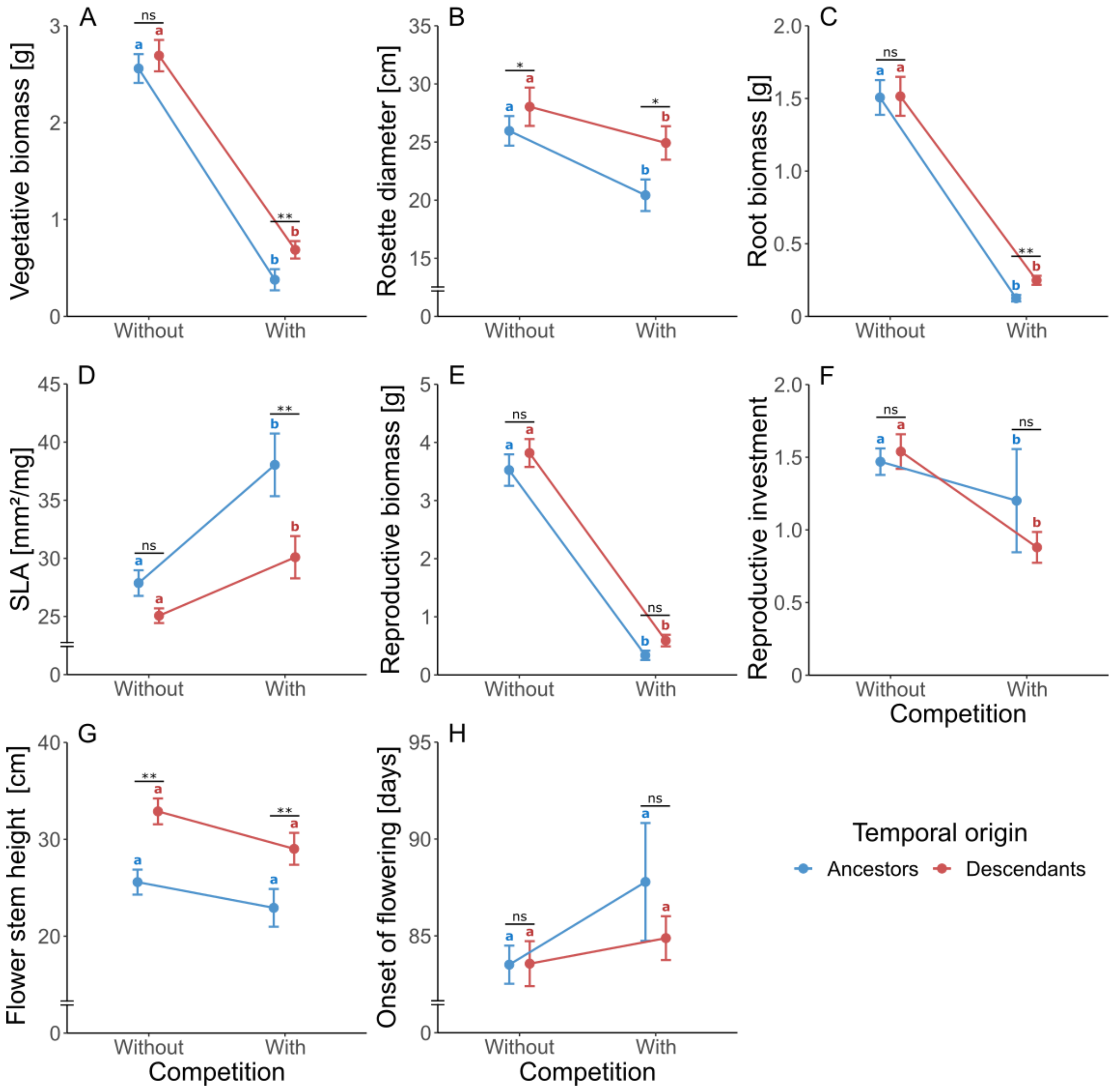

Figure 2. Vegetative biomass (A), rosette diameter (B), root biomass (C), specific leaf area (D), reproductive biomass (E), reproductive investment (F), flower stem height (G) and onset of flowering (H) of ancestors (blue) and descendants (red) of *Leontodon hispidus* grown either without competition or with competition. Shown are reaction norms connecting the means of the competition treatments with their standard errors. Significant differences between ancestors and descendants in each treatment are indicated with asterisks (p > 0.05 ns; p = 0.05 – 0.01 *; p = 0.01 – 0.001 **). Significant differences (p < 0.05) between competition treatments are shown by different letters in their respective colour for each temporal origin (blue letters for ancestors and red letters for descendants).

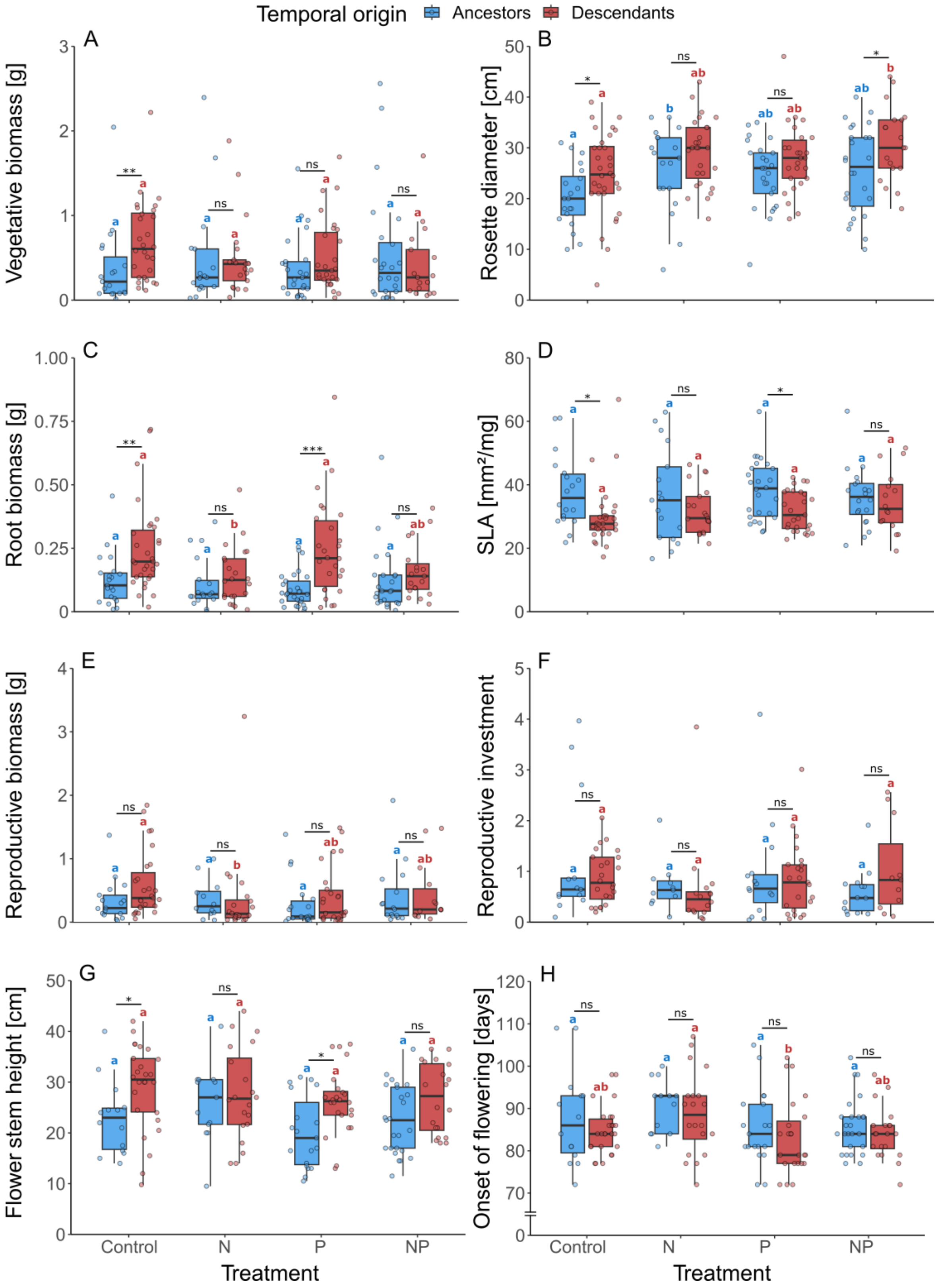

Figure 3. Vegetative biomass (A), rosette diameter (B), root biomass (C), specific leaf area (D), reproductive biomass (E), reproductive investment (F), flower stem height (G) and onset of flowering (H) of ancestors (blue) and descendants (red) of *Leontodon hispidus* grown under different nutrient treatments (control, N, P, NP). Shown are boxplots with the raw data as scatter points. Significant differences between ancestors and descendants in each treatment are indicated with asterisks ($p > 0.05$ ns; $p = 0.05 – 0.01$ *; $p = 0.01 – 0.001$ **; $p < 0.001$ ***). Significant differences ($p < 0.05$) between nutrient treatments are shown by different letters in their respective colour for each temporal origin separately (blue letters for ancestors and red letters for descendants).

Table 1. Results of the statistical models testing the effects of temporal origin (ancestors, descendants), competition (with, without) and their interaction on the response variables (y) vegetative biomass, rosette diameter, root biomass, specific leaf area (SLA), reproductive biomass, reproductive investment, flower stem height and onset of flowering of *Leontodon hispidus*. We used linear mixed-effects models followed by *Anova* (Type 1). Response variables were transformed if needed to fulfil model assumptions. Shown are degrees of freedom (*df*), *F* values and *p* values with significant *p* values (< 0.05) in bold.

Response variable	Transformation	Explanatory variable	*df*	*F* value	*p* value
Vegetative biomass	sqrt(y)	Origin	1	7.12	**0.050**
		Competition	1	281.70	< **0.001**
		Origin × Competition	1	3.93	**0.038**
Rosette diameter	$(y)^3$	Origin	1	12.73	< **0.001**
		Competition	1	17.15	< **0.001**
		Origin × Competition	1	0.06	0.800
Root biomass	log(y)	Origin	1	4.06	0.061
		Competition	1	325.85	< **0.001**
		Origin × Competition	1	11.83	< **0.001**
SLA	log(y)	Origin	1	10.07	**0.007**
		Competition	1	26.97	< **0.001**
		Origin × Competition	1	2.49	0.118
Reproductive biomass	y	Origin	1	0.83	0.377
		Competition	1	188.08	< **0.001**
		Origin × Competition	1	0.002	0.965
Reproductive investment	log(y)	Origin	1	0.03	0.863
		Competition	1	24.88	< **0.001**
		Origin × Competition	1	0.03	0.862
Flower stem height	y	Origin	1	10.91	**0.004**
		Competition	1	5.54	**0.021**
		Origin × Competition	1	0.01	0.934
Onset of flowering	y	Origin	1	0.97	0.339
		Competition	1	3.79	0.054
		Origin × Competition	1	1.05	0.309

Table 2. Results of the statistical models testing the effects of temporal origin (ancestors, descendants), nutrient treatment (control, N, P, NP) and their interaction on the response variables (y) vegetative biomass, rosette diameter, root biomass, specific leaf area (SLA), reproductive biomass, reproductive investment, flower stem height and onset of flowering of *Leontodon hispidus*. We used linear mixed effects models followed by *Anova* (Type 1). Response variables were transformed if needed to fulfil model assumptions. Shown are degrees of freedom (*df*), *F* values and *p* values with significant *p* values (< 0.05) in bold.

Response variable	Transformation	Explanatory variable	*df*	*F* value	*p* value
Vegetative biomass	log(y)	Origin	1	3.54	0.081
		Nutrients	3	0.73	0.533
		Origin × Nutrients	3	1.31	0.274
Rosette diameter	y	Origin	1	6.58	**0.024**
		Nutrients	3	6.18	**< 0.001**
		Origin × Nutrients	3	0.44	0.722
Root biomass	sqrt(y)	Origin	1	11.53	**0.004**
		Nutrients	3	1.91	0.129
		Origin × Nutrients	3	3.03	**0.031**
SLA	log(y)	Origin	1	5.25	**0.038**
		Nutrients	3	0.96	0.415
		Origin × Nutrients	3	0.84	0.476
Reproductive biomass	log(y)	Origin	1	0.83	0.374
		Nutrients	3	2.31	0.079
		Origin × Nutrients	3	1.21	0.307
Reproductive investment	log(y)	Origin	1	0.00	0.965
		Nutrients	3	1.24	0.299
		Origin × Nutrients	3	0.82	0.484
Flower stem height	y	Origin	1	8.83	**0.009**
		Nutrients	3	1.80	0.149
		Origin × Nutrients	3	1.01	0.392
Onset of flowering	y	Origin	1	1.66	0.219
		Nutrients	3	3.55	**0.016**
		Origin × Nutrients	3	0.06	0.982

Supplement material

Table S1. Chemical composition of soil samples taken at the collection sites of our study species *Leontodon hispidus*. We took four samples of 25 cm² soil each at 10 cm depth at random positions and mixed them together. The samples were analyzed to determine the amount of fundamental minerals (total element content of P, K, S, Ca, total C, N and S), as well as pH level and salinity.

pH	Salinity [µS/cm]	N [%]	C [%]	TIC [%]	C org [%]	C/N	S [%]	Ca [mg/kg]	K [mg/kg]	Mg [mg/kg]	P [mg/kg]
6.46	88	0.49	6.90	0.00	6.90	14.14	0.01	3966	8608	1090	530

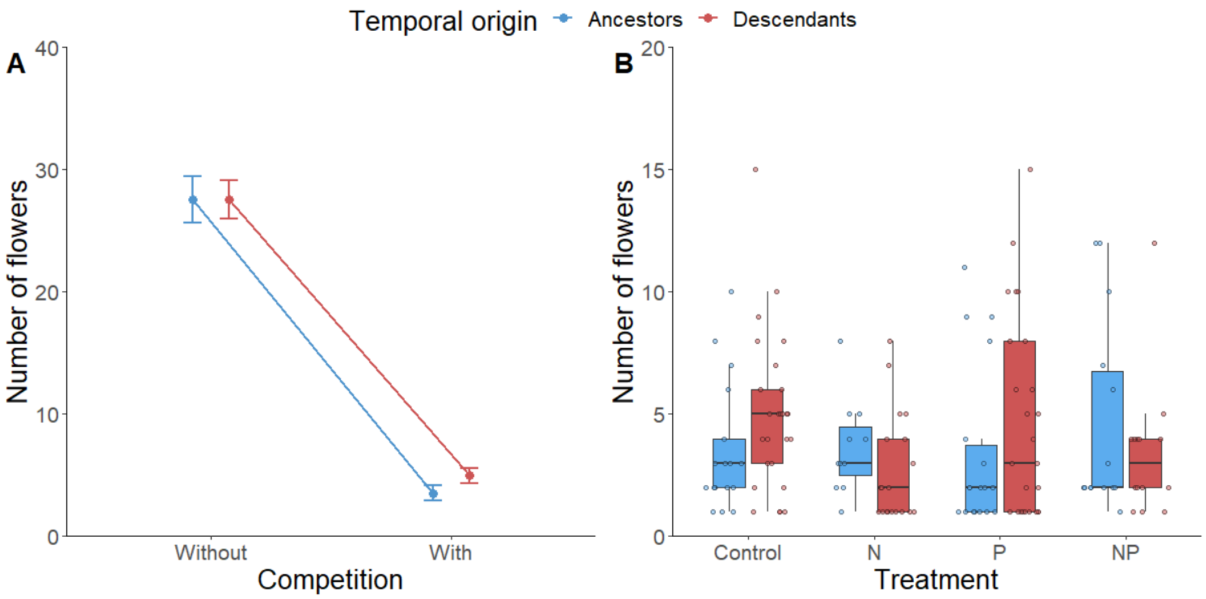

Figure S1. Number of flowers of ancestors (blue) and descendants (red) of *Leontodon hispidus* grown either without competition or with competition (A) and under different nutrient treatments (B). Shown are means and standard errors for (A) and boxplots with raw data as scatter points for (B).