Using the resurrection approach to investigate rapid plant adaptation to recent environmental changes

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Pascal Karitter
from Saarlouis, Germany

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Dean

Prof. Dr. Sven Klimpel
Johann Wolfgang Goethe-Universität Frankfurt am Main
Institut für Ökologie, Evolution und Diversität
Integrative Parasitologie und Tierphysiologie
Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Deutschland

Thesis examiners

Prof. Dr. J. F. Niek Scheepens
Johann Wolfgang Goethe-Universität Frankfurt am Main
Institut für Ökologie, Evolution und Diversität
Evolutionäre Ökologie der Pflanzen
Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Deutschland

Ass. Prof. Dr. Silvia Matesanz
Rey Juan Carlos University
Department of Biology and Geology Physics and Inorganic Chemistry
Biodiversity and Conservation
C/Tulipán s/n, 28933 Móstoles, Madrid, Spain

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Summary

Anthropogenic activities have a major impact on our planet and rapidly drive biodiversity loss in ecosystems at a global scale. Particularly over the last century, rising CO_2 emissions significantly raised global temperatures and increased the intensity and frequency of droughts and heatwaves. Additionally, 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. It is important to understand how these rapid changes affect the evolution of plant populations and their adaptive potential. Adaptation by natural selection (i.e., adaptive evolution) within a few generations is an essential process as a response to rapid environmental changes. Rapid evolution of plant populations can be detected by using the so-called resurrection approach. Here, diaspores (i.e., seeds) from a population are collected before (ancestors) and after (descendants) a potential selection pressure (e.g., consecutive years of drought or changes in nutrient supply). Comparing phenotypes of ancestors and descendants in a common environment such as an outside garden, greenhouse, or climate chamber, may then reveal evolutionary changes. Ideally, plants are first grown in a common environment for an intermediate refresher generation to reduce parental and storage effects.

The aim of this thesis was to investigate the occurrence of adaptive evolution in natural plant populations in response to rapidly changing environments over the past three decades. I conducted three experiments using the resurrection approach to generate comprehensive data on the adaptive processes that acted on three plant populations from three different species over the last three decades. Furthermore, I filled knowledge gaps in plant evolutionary ecology and conceptually developed the resurrection approach further.

In **Chapter I**, I performed a novel approach by testing for adaptive evolution in natural plant populations using the resurrection approach in combination with *in-situ* transplantations. I 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, I sowed seeds of ancestors and descendants of two species (*L. hispidus* and *C. vulgare*) to the collection sites in order to investigate germination rates and establishment. In transplanted *M. ciliata* plants, I observed lower mortality and larger plant size in descendants compared to ancestors. This suggests that descendants are better adapted than ancestors to the current environmental conditions, which were exceptionally hot and dry during the study period. Seedlings of *C. vulgare* descendants tended to be smaller, and seedlings of L*. hispidus* descendants produced fewer leaves compared to their ancestors in their contemporary environmental conditions. In *C. vulgare* and *L. hispidus*, I found evidence for the evolution towards faster germination. Moreover, descendant seeds of *C. vulgare* were better adapted to the unfavorable conditions during the experimental period. In conclusion, **Chapter I** demonstrates that the novel approach of combining resurrection ecology with transplant experiments is a promising avenue to rigorously test for adaptive evolution in changing environments.

In **Chapter II**, I investigated whether the common calcareous grassland herb *Leontodon hispidus* recently evolved its competitive ability and response to nutrient availability. I 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, I applied nutrient treatments to plants grown under competition, supplying plants weekly with either no fertilizer, or with nitrogen, phosphorus, or both. I found evidence for the 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, I observed evolution of taller flower stems, which might reflect a strategy to increase pollinator visits under the existing pollinator decline in recent decades. **Chapter II** demonstrates rapid contemporary evolution of competitive ability, but also the complexity of the underlying processes of contemporary evolution, and sheds light on the importance of understudied selection agents in the resurrection approach, such as nutrient availability.

In **Chapter III**, I assessed the reproducibility of phenotypic differences between genotypes among three different growth facilities (climate chamber, greenhouse, and outdoor garden). I also evaluated differences in phenotypic expression between plants grown after one vs. two intermediate generations (i.e., refresher generations). I performed this experiment within the framework of the resurrection approach and compared ancestors and descendants of the same population of *Leontodon hispidus*. I observed very strong differences among plants growing in the different growth facilities. I found a significant interaction between the growth facility and the temporal origin (ancestors vs. descendants): descendants had significantly larger rosettes than ancestors only in the greenhouse and they flowered significantly later than ancestors exclusively in the climate chamber. I did not find significant differences between intermediate generations within the growth facilities. Overall, **Chapter III** shows that the use of a particular experimental system can dictate the presence and magnitude of phenotypic differences. This implies that absence of evidence is not evidence of absence when it comes to investigating genetically based trait differentiation among plant origins (in space or time). Experimental systems should be carefully designed to provide meaningful conditions, ideally mimicking the environmental conditions of the population's origins. Finally, growing a second intermediate generation did not impact the genetic differences of ancestors and descendants within the environments, supporting the idea that only one intermediate generation may be sufficient to reduce detectable parental and storage effects.

The resurrection approach allows a better understanding of rapid plant adaptation, but some limitations deserve to be highlighted. I only studied one population per species, and **Chapters II** and **III** only focus on one population of *L. hispidus*, which is also hampering generalizations, as adaptive potential can vary greatly among populations of the same species. I only compared the ancestral genotypes to one descendant sample with a long time span in between (26 – 28 years), which makes it hard to pinpoint the selection agents that caused the genetic differentiation among the sampling years. Hence, closely monitoring biotic and abiotic factors of the studied populations between the ancestral and descendant sampling in future studies, would make identifying the responsible selection pressures more precise. I also recommend sampling multiple populations over consecutive years to improve the robustness of results and make generalizations more approachable.

Furthermore, combining the resurrection approach with other methods such as *in-situ* transplantations will be valuable to offset the limitation that adaptations cannot be proven under artificial conditions (e.g., in the greenhouse).

The series of experimental studies presented in this thesis is a valuable contribution to the field of evolutionary biology with input from the novel, innovative approach of resurrection ecology. I demonstrated that the incorporation of *in-situ* transplantations into the resurrection approach is a vital step to provide firm evidence for the adaptive evolution of plant populations. Here, especially seed traits showed high adaptive potential and will be interesting to study in more detail in the future to unveil general patterns of adaptations in this crucial life cycle stage of plants. Furthermore, I explored evolutionary changes in the competitive ability of *L. hispidus* in response to a selection agent, nutrient availability, which is understudied within the resurrection approach. I found evidence for the evolution of competitive ability in one of my study species, which may be explained by changes in nutrient availability, but the results also suggest that pollinator decline might be an important factor to consider for the evolution of pollinator-dependent plant populations. Finally, I generated valuable information for the methodological planning of resurrection studies with regard to the choice of the growth facility and the needed number of intermediate generations. I showed that the choice of the growth facility and its environmental conditions can greatly impact the expression of phenotypic differences among genotypes. Therefore, careful consideration of the experimental environment is vital to ensure that valid conclusions are drawn from the experiment. Furthermore, the results on *L. hispidus* suggest that only one intermediate generation would be sufficient to reduce detectable parental and storage effects, which is especially useful when working with perennials, but such effects may be species-dependent. Overall, my work demonstrates rapid evolution of multiple plant populations and provides the first steps in new directions for the resurrection approach. Despite some methodological limitations, future studies can greatly benefit from the insights generated in the presented studies and can continue to strengthen our understanding of rapid plant evolution in response to the complex selection pressures that plant populations face in this era of global change.

Zusammenfassung

Anthropogene Aktivitäten haben einen erheblichen Einfluss auf unseren Planeten und treiben den schnellen Verlust der Biodiversität in Ökosystemen weltweit voran. Insbesondere im Laufe des letzten Jahrhunderts haben steigende CO_2-Emissionen die globalen Temperaturen signifikant erhöht und die Intensität sowie Häufigkeit von Dürren und Hitzewellen sind angestiegen. Zusätzlich tragen landwirtschaftliche Flächennutzung und die Verbrennung fossiler Brennstoffe durch umfangreiche Düngung und Ablagerung aus der Atmosphäre kontinuierlich zur Freisetzung von Stickstoff (N) und Phosphor (P) in Ökosysteme bei, was wiederum Pflanzenpopulationen beeinflusst. Es ist von hoher Bedeutung, die Auswirkungen dieser raschen Veränderungen auf Pflanzenpopulationen und deren Anpassungspotenzial zu verstehen. Die Anpassung durch Evolution (d.h. natürliche Selektion) als Reaktion auf schnelle Umweltveränderungen ist ein bedeutender Prozess, der auch schnell und innerhalb weniger Generationen stattfinden kann. Die schnelle Evolution von Populationen kann durch den sogenannten *Resurrection Approach* nachgewiesen werden. Hierbei werden Diasporen (d.h. Samen) einer Population vor (Vorfahren) und nach (Nachfahren) eines potenziellen Selektionsdrucks (z.B. Dürre oder Veränderungen in der Nährstoffverfügbarkeit) gesammelt. Der Vergleich der Phänotypen dieser beiden Generationen unter gleichen Bedinungen kann dann evolutionäre Veränderungen aufzeigen. Es ist jedoch wichtig, eine Zwischengeneration zu kultivieren, bevor das Experiment durchgeführt wird, um Parental- und Lagereffekte zu reduzieren. Der *Resurrection Approach* kann in verschiedenen Kultivierungseinrichtungen durchgeführt werden (z.B. Garten, Gewächshaus, Klimakammer), die sich in ihren Umweltbedingungen erheblich voneinander unterscheiden können. Diese Unterschiede können die Präsenz und Größe phänotypischer Unterschiede beeinflussen. Daher ist unklar, ob Ergebnisse aus diesen Experimenten in verschiedenen Kultivierungseinrichtungen reproduzierbar sind.

Das Ziel dieser Dissertation war es, zu untersuchen, wie Pflanzen in ihren evolutionären Anpassungen von sich ändernden Umweltbedingungen der letzten Jahrzehnte beeinflusst wurden. Ich führte mehrere Experimente im Rahmen des *Resurrection Approachs* durch, um umfassende Daten zu den evolutionären

Prozessen von drei Pflanzenpopulationen verschiedener Arten über die letzten drei Jahrzehnte zu generieren. Darüber hinaus schloss ich Wissenslücken in der aktuellen pflanzlichen evolutionären Ökologie und entwickelte den *Resurrection Approach* konzeptionell weiter.

In **Kapitel I** führte ich einen neuartigen Ansatz durch, indem ich evolutionäre Anpassungen in natürlichen Pflanzenpopulationen mittels des *Resurrection Approachs* in Verbindung mit *in-situ*-Transplantationen untersuchte. Ich zog Sämlinge von Vorfahren (23 – 26 Jahre alt) und heutigen Nachfahren von drei mehrjährigen Arten (*Melica ciliata, Leontodon hispidus* und *Clinopodium vulgare*) aus kalkhaltigen Magerrasen im Gewächshaus heran und transplantierte sie zurück an ihre Sammelstellen. Zusätzlich säte ich Samen von Vorfahren und Nachfahren von zwei Arten (*L. hispidus* und C*. vulgare)* an den Sammelstellen aus, um Keimraten und Etablierung zu untersuchen. Bei den transplantierten Pflanzen von *M. ciliata* beobachtete ich eine geringere Sterblichkeit und größere Pflanzengröße bei den Nachfahren im Vergleich zu den Vorfahren. Dies deutet darauf hin, dass die Nachfahren besser an die aktuellen Umweltbedingungen angepasst sind, die sich während des Untersuchungszeitraums als außergewöhnlich heiß und trocken erwiesen. Die Nachfahren von *C. vulgare* neigten dazu, kleiner zu sein, und die Nachfahren von *L. hispidus* bildeten in ihren heutigen Umweltbedingungen weniger Blätter im Vergleich zu ihren Vorfahren. Bei *C. vulgare* und *L. hispidus* konnte ich eine Evolution in Richtung schnellerer Keimung feststellen, wobei insbesondere die Samen der Nachfahren von *C. vulgare* besser an die ungünstigen Bedingungen während des experimentellen Zeitraums angepasst waren. Zusammenfassend zeigt **Kapitel I**, dass der neuartige Ansatz, den *Resurrection Approach* mit Transplantationsexperimenten zu kombinieren, einen vielversprechenden Weg darstellt, um evolutionäre Anpassungen in sich verändernden Umgebungen eingehend zu testen.

In **Kapitel II** untersuchte ich, ob eine Population von *Leontodon hispidus* in jüngster Zeit ihre Konkurrenzfähigkeit und ihre Reaktion auf die Nährstoffverfügbarkeit evolutionär angepasst hat. Ich zog Vorfahren, die 1995 gesammelt wurden, und Nachfahren, die 2018 aus einer einzigen Population stammen, unter gemeinsamen Bedingungen heran und unterzog sie einer Konkurrenzbehandlung unter Verwendung des natürlichen Konkurrenten *Brachypodium pinnatum*. Darüber hinaus führte ich

Nährstoffbehandlungen bei unter Konkurrenz wachsenden Pflanzen durch, indem ich Pflanzen wöchentlich entweder ohne Dünger oder mit Stickstoff, Phosphor oder beidem versorgte. Ich fand Hinweise auf Evolution von erhöhter Konkurrenzfähigkeit, da Nachfahren unter Konkurrenzbedingungen mehr vegetative Biomasse produzierten als Vorfahren. Die Konkurrenzfähigkeit hing auch von der Nährstoffbehandlung ab, was darauf hinweist, dass Nachkommen möglicherweise an niedrigere Stickstoffkonzentrationen angepasst sind, was mit dem Rückgang der Stickstoffemissionen in die Atmosphäre seit den 1990er Jahren zusammenhängen könnte. Darüber hinaus beobachtete ich Evolution von höheren Blütenstielen, was eine Strategie sein könnte, um die Besuche von Bestäubern in Anbetracht des existierenden Rückgangs von Bestäubern in den letzten Jahrzehnten zu erhöhen. **Kapitel II** zeigt eine rasche zeitgenössische Evolution der Konkurrenzfähigkeit, verdeutlicht jedoch auch die Komplexität der zugrunde liegenden Prozesse der raschen Evolution und beleuchtet die Bedeutung der im Rahmen des *Resurrection Approachs* wenig erforschten Selektionsfaktoren wie der Nährstoffverfügbarkeit.

Im **Kapitel III** bewertete ich die Auswirkungen von drei verschiedenen Kultivierungseinrichtungen (Klimakammer, Gewächshaus und Außengarten) auf die phänotypischen Unterschiede zwischen Vor- und Nachfahren. Ich untersuchte zudem Unterschiede in der phänotypischen Expression zwischen Pflanzen, die nach einer bzw. zwei Zwischengenerationen gewachsen sind. Ich führte dieses Experiment im Rahmen des *Resurrection Approachs* durch und verglich Vorfahren und Nachfahren derselben Population von *Leontodon hispidus*. Ich beobachtete sehr starke Unterschiede bei Pflanzen, die in verschiedenen Kultivierungseinrichtungen wuchsen. Ich fand eine signifikante Interaktion zwischen der Kultivierungseinrichtung und dem zeitlichen Ursprung (Vorfahren *vs.* Nachfahren): Nachfahren hatten signifikant größere Rosetten als Vorfahren nur im Gewächshaus, und sie blühten signifikant später als Vorfahren ausschließlich in der Klimakammer. Ich fand keine signifikanten Unterschiede zwischen den Zwischengenerationen innerhalb der experimentellen Umgebungen. Insgesamt zeigt **Kapitel III**, dass die Verwendung einer bestimmten Kultivierungseinrichtung die Existenz und Größe phänotypischer Unterschiede beeinflussen kann. Dies impliziert, dass das Fehlen von Beweisen nicht als Beweis für das Fehlen betrachtet werden sollte, wenn es darum geht, genetisch bedingte Unterschiede in Merkmalsausprägungen zwischen Pflanzenursprüngen (im Raum

oder in der Zeit) zu untersuchen. Kultivierungseinrichtungen sollten sorgfältig gestaltet sein, um aussagekräftige Bedingungen zu bieten, die, je nach Forschungsfragen, idealerweise die Umweltbedingungen der Ursprünge der Population nachahmen. Letztlich hatte das Kultivieren einer zweiten Zwischengeneration keinen Einfluss auf die genetischen Unterschiede zwischen Vor- und Nachfahren innerhalb der Kultivierungseinrichtungen, was darauf hindeutet, dass möglicherweise eine einzige Zwischengeneration ausreicht, um nachweisbare Parental- und Lagereffekte zu reduzieren.

Der *Resurrection Approach* ermöglicht ein besseres Verständnis schneller evolutionärer Pflanzenanpassungen, jedoch sind einige Einschränkungen hervorzuheben. Ich habe nur eine Population pro Art untersucht, und **Kapitel II** sowie **Kapitel III** konzentrieren sich nur auf eine Population von *L. hispidus*, was ebenfalls Verallgemeinerungen erschwert, da das adaptive Potenzial zwischen Populationen derselben Art stark variieren kann. Zudem habe ich nur die genetischen Ausprägungen der Vorfahren mit einer langen Zeitspanne dazwischen (26 – 28 Jahre) mit einer einzigen Nachfahrenprobe verglichen, was es schwierig macht, die Selektionsfaktoren zu identifizieren, welche die genetische Differenzierung zwischen den Probenahmejahren verursacht haben. Die genaue Aufzeichnung von biotischen und abiotischen Faktoren der untersuchten Populationen zwischen den Probenahmen der Vor- und der Nachfahren in zukünftigen Studien würde es daher ermöglichen, den verantwortlichen Selektionsdruck genauer zu bestimmen. Es ist auch empfehlenswert, über aufeinanderfolgende Jahre hinweg mehrere Populationen zu untersuchen, um die Robustheit der Ergebnisse zu verbessern und Verallgemeinerungen zugänglicher zu machen. Darüber hinaus wird es wertvoll sein, den *Resurrection Approach* mit anderen Methoden wie *in-situ*-Transplantationen zu kombinieren, um die Einschränkungen des *Resurrection Approachs* auszugleichen.

In dieser Dissertation präsentierte ich eine Reihe experimenteller Studien, die wichtige Beiträge zum Bereich der Resurrektionsökologie und Evolutionsbiologie geleistet haben. Ich zeigte, dass die Integration von *in-situ*-Transplantationen in den *Resurrection Approach* einen entscheidenden Schritt darstellt, um klare Nachweise für die evolutionäre Anpassung von Pflanzenpopulationen zu liefern. Insbesondere zeigten hierbei Sameneigenschaften ein hohes adaptives Potenzial und werden in

Zukunft besonders interessant sein, um allgemeine Muster der Anpassungen in dieser entscheidenden Lebenszyklusphase von Pflanzen aufzudecken. Des Weiteren erforschte ich evolutionäre Veränderungen der Konkurrenzfähigkeit als Reaktion auf den im *Resurrection Approach* wenig erforschten Selektionsfaktor Nährstoffverfügbarkeit. Ich fand Hinweise auf die Evolution der Konkurrenzfähigkeit bei einer meiner Untersuchungsarten, die mit Veränderungen in der Nährstoffverfügbarkeit in Verbindung stehen könnte. Jedoch könnte auch der Rückgang von Bestäubern ein wichtiger Faktor für die Evolution von bestäuberabhängigen Pflanzenpopulationen sein.

Schließlich generierte ich wertvolle Informationen für die methodische Planung von Experimenten des *Resurrection Approachs* in Bezug auf die Auswahl der experimentellen Bedingungen und die benötigte Anzahl von Zwischengenerationen. Ich zeigte, dass die Auswahl der Kultivierungseinrichtung einen erheblichen Einfluss auf die Expression phänotypischer Unterschiede zwischen den Genotypen haben kann. Eine sorgfältige Berücksichtigung der experimentellen Bedingungen ist entscheidend, um sicherzustellen, dass gültige Schlussfolgerungen aus dem Experiment gezogen werden können. Die Ergebnisse legen außerdem nahe, dass eine einzige Zwischengeneration ausreichen würde, um nachweisbare Parental- und Lagereffekte zu reduzieren, was besonders nützlich ist, wenn mit mehrjährigen Pflanzen gearbeitet wird, jedoch möglicherweise artabhängig ist. Insgesamt demonstrierte meine Arbeit schnelle Evolution mehrerer Pflanzenpopulationen und lieferte erste Schritte in neue Richtungen für den *Resurrection Approach*. Trotz einiger methodischer Einschränkungen können zukünftige Studien erheblich von den Erkenntnissen der vorgestellten Arbeiten profitieren und dazu beitragen, unser Verständnis der schnellen Pflanzenevolution unter den komplexen Selektionsdrücken, denen Pflanzenpopulationen in dieser Ära des globalen Wandels ausgesetzt sind, zu vertiefen.

General Introduction

Rapid environmental changes

Rapid environmental changes have a major impact on our planet and its ecosystems. Anthropogenic activities have significantly increased CO_2 concentrations in the atmosphere over the last century and, as a consequence, raised global temperatures, driven regional and seasonal temperature extremes, and changed precipitation patterns and their global variability (Dore, 2005; IPCC, 2018). According to estimates of the Millennium Ecosystem Assessment (Duraiappah et al., 2005), climate change will probably be the biggest driver of biodiversity loss by the end of the century and will also threaten plant diversity on the intraspecific level. Furthermore, the changes in climate in combination with habitat loss and fragmentation, and pesticide application have caused the steady decline of pollinators over recent decades, which is affecting pollinator-dependent plant populations (Potts et al., 2010). In addition to impacts related to climate, fossil fuel combustion and agricultural land use are causing the continuous release of nitrogen (N) and phosphorus (P) into ecosystems globally through deposition from the atmosphere and extensive fertilization (Galloway et al., 2008; Newman, 1995; Smith et al., 1999). Nutrient enrichment can have implications for plant populations through competitive exclusion, higher susceptibility to pests and abiotic stressors, soil acidification, and even toxicity (Bobbink et al., 2010; Hautier et al., 2009; Johnson, 1993; Olsson & Tyler, 2004; Stevens et al., 2010). Whereas N emissions have been steadily decreasing after their peak in the 1990s (European Environment Agency, 2021), P levels are still higher than 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). It is undisputed that these global environmental changes have already had a strong impact on plant life and will continue to have a strong influence on it in the future. Since most vascular plants are sessile organisms, they cannot respond to environmental changes through movement and will therefore be under increased pressure imposed from these rapid changes. If plant populations are unable to adapt, they are threatened with displacement from their current habitats and, depending on the circumstances, even extinction (Root et al., 2003).

Plant responses to rapid environmental changes

In general, plant responses to novel environmental conditions can be categorized into three different processes: (1) dispersal into more favorable habitats, (2) adaptive evolution, and (3) phenotypic plasticity (Becklin et al., 2016). However, environmental changes may often be too rapid and too severe, causing populations to become locally extinct before they can disperse to more favorable habitats (Root et al., 2003). However, plant populations may persist if they are able to adapt quickly. For adaptive evolution to occur on the population level, three conditions need to be met. First, there must be phenotypic trait variation within the population. Second, this variation needs to be at least partially heritable, and third, certain trait characteristics need to increase fitness (i.e., affecting survival, growth, and/or reproductive success) under selection pressure in a given environment (Darwin, 1859). Natural selection can act in three different ways on a given trait: (1) directional selection, where individuals with trait values at one end of the phenotypic spectrum are selected; (2) stabilizing selection, where individuals with intermediate trait values are selected; (3) disruptive selection, where both ends of the trait spectrum are selected (Endler, 1986).

According to the classical view, evolution is a slow process, but advancements in our understanding of evolutionary biology show that evolution can occur quickly and within one or a few generations (Franks et al., 2007; Thompson et al., 2013). For instance, Thompson and colleagues (2013) have shown that populations of *Thymus vulgaris* have developed adaptive evolution to milder winters since 1970. The number of phenotypes that are sensitive to winter frost has increased significantly as milder winters have eased the selection pressure of frost. In *Arabidopsis lyrata*, strong selection pressure on earlier flowering has been observed in combination with grazing (Sandring et al., 2007). Similarly, Giménez-Benavidez and colleagues (2011) found that current selection pressure on the flowering time of Mediterranean mountain plants was caused by climate change.

Beyond adaptive evolution, phenotypic plasticity is a key mechanism for rapid plant adaptation. Phenotypic plasticity is the ability of a genotype to produce different phenotypes depending on the environment (Sultan, 2000). In general, it is important to distinguish between neutral, adaptive, and maladaptive plasticity. Adaptive plasticity

promotes the establishment and persistence in a new environment, while maladaptive plasticity reduces plant fitness, and neutral plasticity has no effect on fitness (Ghalambor et al., 2007). Strongly plastic species could be limited in their evolutionary potential because they experience a lower selection pressure (Oostra et al., 2018; Price et al., 2003). However, the plasticity of a trait can also evolve: adaptive evolution to environmental changes can take place by increasing the variance of phenotypic responses (Pigliucci, 2005; Scheiner, 1993; Schlichting and Levin, 1986). Since important climatic variables do not only change on average but also become more variable, increased plasticity may be favorable, as was shown in populations of *Senna candolleana* in highly variable climatic environments (Lázaro-Nogal et al., 2015). At the same time, maladaptive plasticity can also be evolutionarily reduced by selection (Ghalambor et al., 2007).

Investigating rapid plant responses

Long-term observation studies have been used to detect rapid plant responses to climate change. They showed early leaf formation (Jeong et al., 2011; Slayback et al., 2003), advanced flowering and fruit formation (Cook et al., 2012; Fitter & Fitter, 2002), in synchrony with regional warming (Menzel et al., 2006; Parmesan, 2007). These observation studies have the disadvantage that they cannot distinguish between plastic and selective causes. In order to disentangle plastic responses from adaptive evolution, experimental approaches are needed. Common-environment experiments are a powerful approach for this purpose and allow to attribute phenotypic differences to a genetic basis by comparing plants from different origins in the same developmental environment (Turesson, 1922). This concept was further expanded into reciprocal transplantation experiments, which allow the detection of local adaptation by transplanting individuals originating from distinct populations reciprocally to all original sites from which they were collected (Clausen et al., 1940; Kawecki and Ebert, 2004). Hence, local adaptation is proven if plants perform best in their local (i.e., home) site in comparison to the foreign (i.e., away) sites. Common-environment experiments can further be used to study rapid evolution of plant populations to recent environmental changes by applying artificial selection pressure over multiple generations (Hill and Caballero, 1992) and to quantify phenotypic plasticity when treatments or multiple transplantation locations are included (Merilä and Hendry, 2014).

Another powerful method to detect rapid evolution of plant populations to recent environmental changes is the so-called resurrection approach (Franks et al., 2018b; Kooyers et al., 2021; Vtipil and Sheth, 2020). This approach consists of an experimental design that uses diaspores (i.e., seeds) from a population collected before (ancestors) and after (descendants) a potential selection pressure (e.g., consecutive years of drought or changes in nutrient supply, Fig. 1). Comparing phenotypes of these two generations in a common environment may then reveal evolutionary changes (Franks et al., 2007). This method is particularly suitable for plants since the seeds of many species can be stored for a long period and can still remain viable (Walters et al., 2005). However, this methodological approach needs additional considerations for its correct implementation. For instance, it is crucial to make sure that the seed collections of ancestors and descendants represent the genetic diversity of the populations (Franks et al., 2018b). Another potential confounding factor can be the so-called “invisible fraction”, i.e., only a fraction of the seeds may survive the storage conditions, which might genetically correlate with post-emergence plant traits (Weis, 2018). If the germination rates of ancestors are high or at least equal to the germination rate of the descendants, the risk of the “invisible fraction” should be low (Weis, 2018). One drawback of resurrection studies is the waiting period after collecting the ancestral seed material in order to detect significant selection of the population (forward-in-time approach), but backward-in-time approaches are also possible if seed material was already fortuitously collected in the past (Franks et al., 2018b). Recently, seeds collected for conservation purposes in seed repositories have been successfully used in resurrection studies, and they may be a valuable resource for future resurrection studies (Ensslin et al., 2023; Everingham et al., 2021; Rauschkolb et al., 2022a; Rauschkolb et al., 2022b). These seed repositories have the advantage that researchers do not have to wait for the populations to evolve to contemporary environmental changes but can simply revisit the populations of the stored seed material and collect descendant seeds. However, it is decisive that the exact sampling locations have been recorded by the seed repository to ensure that the same population is sampled. Finally, dedicated seed banks for resurrection studies (Project Baseline) are being developed on a large scale in the United States with standardized sampling protocols and will provide valuable opportunities for future research in this field (Etterson et al., 2016), and similar projects are likely to emerge in Europe and worldwide.

With the resurrection approach and common-environment experiments in general, it is important to reduce non-genetic effects such as parental or storage effects. Parental plants can significantly influence the phenotype of their seedlings regardless of the genes that are passed on (Auge et al., 2017; Badyaev and Uller, 2009). Parental effects include seed provisioning, epigenetic processes through inheriting DNA methylations or chromatin changes, and hormone-driven effects on physiology (Blödner et al., 2007; Elwell et al., 2011; Herman and Sultan, 2011; Jablonka and Raz, 2009; Richards et al., 2017). Especially mother plants can significantly influence the growth of their offspring by providing their seeds with varying amounts of resources. Such seed provisioning is one major parental effect that can greatly impact the fitness of the offspring since the amount of provisions for the seeds can depend on the general environmental conditions (e.g., light quality and duration) and resource availability of the mother plant (Herman and Sultan, 2011). Furthermore, seed quality can be affected by long storage and lead to reduced germination and post-emergence fitness, which is especially common in the ancestral seed material of resurrection studies (Franks et al., 2007). These biases can be accounted for by acclimating the study plants under common environmental conditions for one or more generations before starting the experiment (Franks et al., 2018b; Kawecki and Ebert, 2004). Parental effects can persist over multiple generations (Wulff et al., 1999), but are often no longer visible after one generation in a common environment (Agrawal, 2002; Gianoli, 2002). Epigenetic effects, however, can be more persistent and will often be still apparent after one intermediate generation and therefore, growing at least two intermediate generations is recommended (Latzel, 2015). Additionally, long-term storage of seed material could also cause carry-over effects into subsequent generations. In this sense, resurrected plants from stored seeds may have lower fitness due to the storage and produce lower-quality seeds as a consequence (Gebeyehu, 2020). Here, a second intermediate generation may also be advisable to control for this possible storage effect.

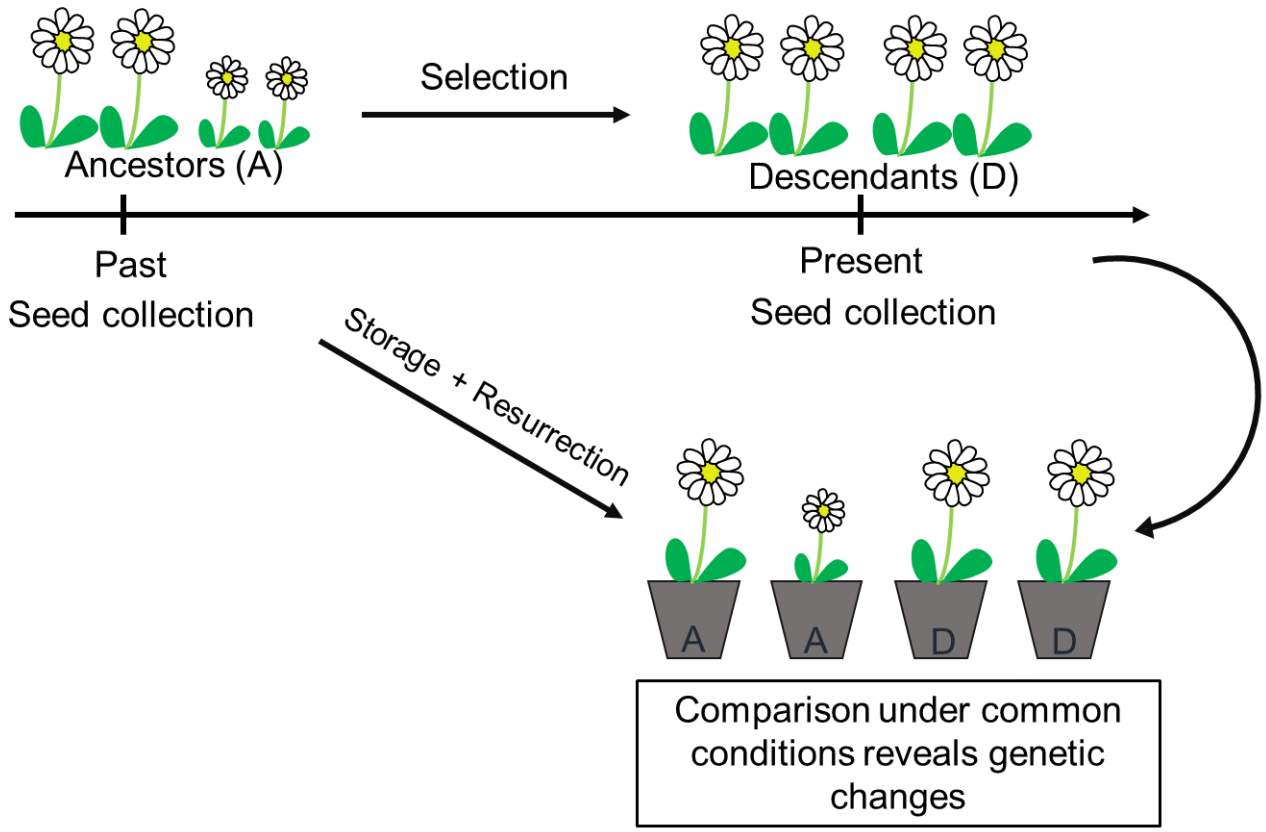

Figure 1. Conceptual visualization of the resurrection approach. Seed material of ancestors (A) is collected and stored in a seed repository. After a waiting period, seeds of descendants (D) of the same population are collected. Stored seeds are resurrected and compared under common conditions, which reveals genetic changes that occurred during the waiting period. Ideally, plants are first grown in an intermediate generation to reduce parental and storage effects.

So far, the resurrection approach has been applied in common-environment experiments such as greenhouse and outdoor gardens and in combination with different treatments (Franks et al., 2007; Horgan-Kobelski et al., 2016; Van Dijk and Hautekèete, 2014). Franks and colleagues (2018) reviewed twelve resurrection studies and found that in all studies, a rapid evolutionary change in one or more characteristics occurred and that these changes could be adaptive. For example, Franks and colleagues (2007) showed that consecutive summer droughts led to early flowering in an annual species *(Brassica rapa*) within only a few generations. Similarly, Van Dijk and Hautekèete (2014) evidenced an evolutionary advancement of the flowering onset in wild turnip (*Beta vulgaris*), which was likely caused by increasing temperatures. Furthermore, adaptive evolution of photosynthesis and reproduction rate to increased drought and sunlight was observed after ten years in the knotweed *Polygonum cespitosum* (Horgan-Kobelski et al., 2016). As a compelling addition to resurrection studies, researchers have included multiple environments or treatments 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; Rauschkolb et al., 2022b). However, some potential selection agents remain understudied. The evolution of competitive ability has been investigated in some resurrection experiments (Frachon et al., 2017; Sultan et al., 2013; Ziska, 2017), but resurrection studies on evolutionary responses to nutrient availability are, to my knowledge, still missing. Since competitive ability also strongly depends on abiotic factors, it is crucial to examine the evolution of competitive ability in the context of changing nutrient availability.

Resurrection studies can be performed in a greenhouse, outside garden, or climate chamber (i.e., growth chamber). These growth facilities can differ greatly from natural conditions. For instance, air temperature and water and nutrient availability are often much more benign under controlled conditions compared to field conditions (Poorter et al., 2016). Moreover, temporal dynamics of soil water availability and air temperature, typical in natural conditions, are very challenging to realistically implement under experimental conditions. Especially the greenhouse, which is commonly used for resurrection studies (e.g., Anstett et al., 2021; Franks et al., 2007; Gay et al., 2022; Hamann et al., 2018; Lambrecht et al., 2020), can create very high temperatures depending on the ventilation and is known to cause shading due to the structural elements (Cabrera-Bosquet et al., 2016). Furthermore, experimental conditions for common-environment experiments can vary greatly from each other (e.g., greenhouse and outside garden), and genotype-environment interactions due to plastic responses to the environment in physiology, metabolism, and growth, and the genetic variation of these traits among genotypes can greatly affect the results (Des Marais et al., 2013). It has been shown that phenotypic differences among genotypes can be lower under benign conditions (i.e., optimal lab conditions) compared to more stressful conditions (Stanton et al., 2000), meaning that comparisons of the phenotype may not always reveal genetic differentiation. Therefore, results from common-environment experiments may not be consistent throughout different experimental environments (e.g., growth facilities) and may not be accurately reproduced. As a consequence, we may miss evidence of relevant genetic variation, or, in contrast, we may detect genetic variation that currently has no adaptive relevance under natural conditions. Since we cannot be sure how the fitness of plants in *ex-situ* cultivation relates to fitness under field conditions, resurrection studies performed in controlled

conditions cannot deliver firm proof of adaptive evolution. Consequently, resurrection studies should be combined with *in-situ* transplantations to the sites of population origins (Franks et al., 2018b). Ancestors and descendants need to be transplanted to the exact location where they were sampled, and by doing so, the strength of adaptation of the ancestral and descendant populations to the current environment can be measured.

Study system

For my study system I chose the vegetation of calcareous grasslands. These habitats are usually nutrient-poor and often grazed by sheep or managed through mowing. They harbor the largest number of vascular plant species in Central Europe and are strongly affected by rapid environmental changes (Wilson et al., 2012). Calcareous grasslands have steadily declined over the 20th century due to changes in land use towards arable fields, forestry, and high-production grasslands (Poschlod and WallisDeVries, 2002). Beyond habitat fragmentation and droughts, calcareous grasslands are accumulating nutrients due to atmospheric deposition or fertilization of adjacent agricultural fields, threatening many species that are adapted to nutrient-poor soil conditions (Smits et al., 2008). Many plant populations in calcareous grasslands may not be adapted to these stressors and thus need to adapt rapidly (Becklin et al., 2016), making this habitat an ideal study system for experiments on rapid evolution.

I selected three perennial plants that are common species in calcareous grasslands and widespread across Europe (Fig. 2): *Melica ciliata* L. (Poaceae), *Clinopodium vulgare* L. (Lamiaceae) and *Leontodon hispidus* L. (Asteraceae). *Melica ciliata* is a perennial grass and hemicryptophyte, that is commonly found in nutrient-poor calcareous or scree grasslands. It typically flowers in June and is wind-pollinated. It can reproduce vegetatively or sexually via seeds (Kühn and Klotz, 2002). *Clinopodium vulgare* is a herbaceous, perennial hemicryptophyte (Düll and Kutzelnigg, 2016; Parolly and Rohwer, 2016). This species grows in dry grasslands, shrubberies, and forest edges with typical flowering onset from July to September. It is pollinated through selfing or by bumblebees and butterflies (Düll and Kutzelnigg, 2016; Eggenberg et al., 2018; Kofidis et al., 2007; Parolly and Rohwer, 2016). *Leontodon hispidus* is a perennial rosette-forming herbaceous plant typical in dry or semi-dry

calcareous grasslands (Kühn and Klotz, 2002). It is self-incompatible and can flower in the first year after germination, which typically occurs from June to October. It is able to reproduce vegetatively or sexually by seed (Kühn and Klotz, 2002).

Figure 2. Pictures of the study species *Melica ciliata* (A), *Clinopodium vulgare* (B), and *Leontodon hispidus* (C). Pictures are taken from the public domain.

Seed material and collection sites

For each species, one specific population was selected from a calcareous grassland within Belgian nature reserves (Fig. 3, Table 1). The population of *M. ciliata* is located in the nature reserve “Tienne Breumont”, which consists of a hill with a central plateau and is managed by sheep grazing. The population of *C. vulgare* is on a slope in the nature reserve “Tienne du Bi”, which is managed by yearly mowing and cutting thickets. The study population of *L. hispidus* can be found in the nature reserve “Thier à la Tombe” along a slope, and is managed by sheep grazing. The chosen populations are characterized by a high degree of isolation, thereby minimizing the potential for gene flow with other neighboring populations. The proximity to the nearest population of the same species varies, with distances of 1.1 km for *M. ciliata*, 4.1 km for *C. vulgare*, and 1.9 km for *L. hispidus*.

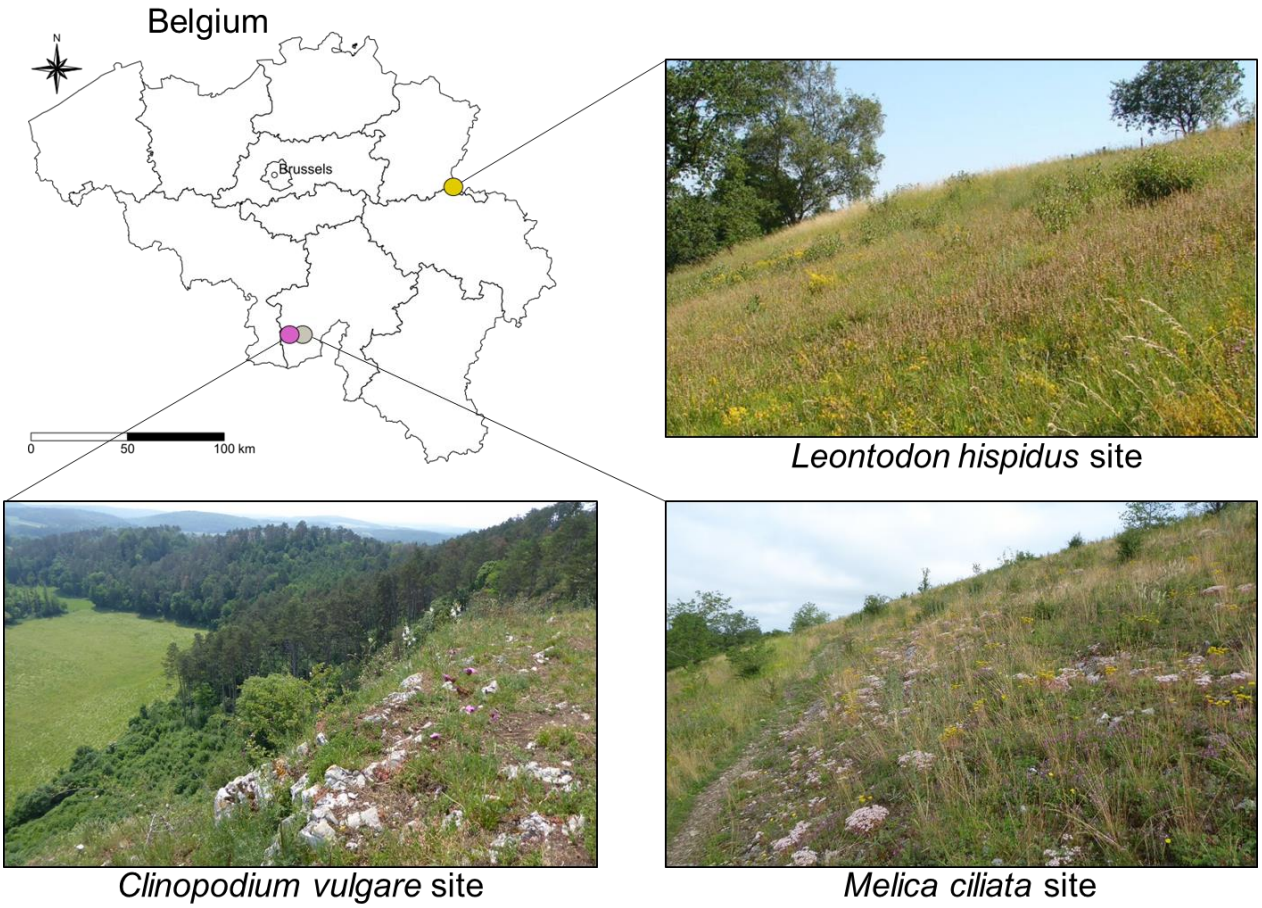

Figure 3. The location of the study populations of *Leontodon hispidus* (yellow), *Clinopodium vulgare* (purple), and *Melica ciliata* (grey) in Belgium. Shown are pictures of the corresponding habitats taken by Sandrine Godefroid.

Ancestral seeds were originally collected in the years 1992 and 1995, while descendant seeds were gathered in 2018 and 2020, depending on the species. It is noteworthy that *C. vulgare* and *L. hispidus* are capable of reproducing within the first year, whereas *M. ciliata* typically requires a second year for reproduction. This distinction in reproductive timing sets an upper limit on the number of generations between the seed collections, resulting in up to 23 generations for *C. vulgare*, up to 26 generations for *L. hispidus*, and up to 15 generations for *M. ciliata* between the years of seed collection. The initial seed collections from the ancestors were carried out by the seed bank staff at the Meise Botanic Garden in Belgium, primarily for conservation purposes. While an effort was made to maximize the representation of the population, specific individual counts of the sampled mother plants were not recorded. Subsequently, all collected seeds were subjected to cleaning, bulking, drying at 15% relative humidity, and long-term storage at -20°C within the seed bank at the Meise Botanic Garden. In the summer of 2018, seeds were obtained from 20 – 47

mother plants within the exact same populations for both *C. vulgare* and *L. hispidus*. These seeds were also subjected to cleaning and bulking before being stored at 4°C. Seeds from *M. ciliata* were collected in 2020 by the Meise Botanic Garden, utilizing the same sampling protocol as was applied in 2018 for *C. vulgare* and *L. hispidus*.

Table 1. Coordinates, management of the nature reserve, collection years, number of collected seed families, and estimated size of the study population for each species.

Species	Coordinates	Management	Collection years	Number of seed families of descendants	Estimated population size in 2018
Melica ciliata	50° 04' 34" N 4°32'35"E	sheep grazing	1992/2020	21	25
Clinopodium vulgare	50°03'55"N 4°26'40"E	mowing	1992/2018	47	500
Leontodon hispidus	50°47'35"N 5°40'25"E	sheep grazing	1995/2018	20	100

Intermediate refresher generations

To mitigate the potential influence of environmental, parental, and storage factors, an intermediate generation was cultivated for ancestors and descendants (hereafter referred to as 'temporal origins') under controlled greenhouse conditions (Rauschkolb et al., 2022b). In this controlled environment, 200 – 300 seeds from each temporal origin were sown. For each temporal origin, 15 seedlings were randomly selected to be cultivated. To prevent inadvertent cross-pollination between the two temporal origins, individuals from each temporal origin were maintained within separate enclosures (Rauschkolb et al., 2022b). As soon as the plants flowered, they were pollinated randomly by hand using pollen derived from individuals of the same temporal origin. However, due to insufficient seed production of *L. hispidus*, I grew a second intermediate generation using bumblebees for pollination. Growing an intermediate generation for *M. ciliata* was unsuccessful, as the plants did not initiate flowering. Consequently, for this particular species, I proceeded with the ancestral and descendant seed material without implementing an intermediate generation in the experimental design.

Objectives

The general aim of my PhD project was to investigate how plants have adapted to changing environmental conditions over the last decades. I conducted three experiments using the resurrection approach to generate comprehensive data on the evolutionary processes of three plant populations from *Melica ciliata*, *Clinopodium vulgare*, and *Leontodon hispidus* over the last three decades (Fig. 4). With these studies, I filled knowledge gaps in current plant evolutionary ecology and conceptually developed the resurrection approach further.

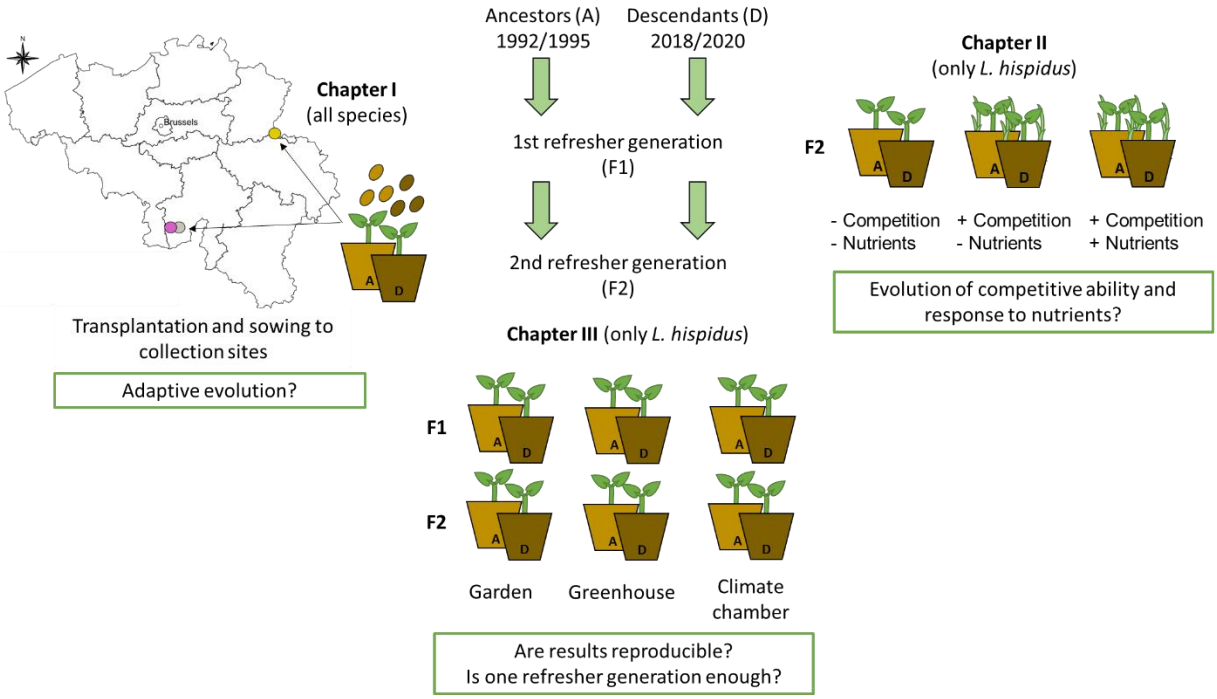

Figure 4. Experimental designs of all three chapters. **In Chapter I** (left), I transplanted seedlings (all species) and sowed seeds (*Leontodom hispidus* and *Clinopodium vulgare)* from ancestors and descendants into the original sampling location to study adaptive evolution. In **Chapter II** (right), I compared ancestors and descendants *of L. hispidus* with competition and nutrient treatments to study recent adaptive evolution to these potential drivers. In **Chapter III** (bottom), I compared ancestors and descendants of *L. hispidus* in multiple growth facilities to test the reproducibility of the phenotypic differentiation, and I compared the effect of multiple refresher (i.e., intermediate) generations.

In **Chapter I**, I investigated recent adaptive evolution of all three study species by conducting a resurrection study in combination with *in-situ* transplantations. I transplanted seedlings and sowed seeds from ancestors and descendants in the original sampling location. There, I measured mortality as an inherent component of individual fitness and a direct indicator of selection pressures acting on plant

populations. Furthermore, I assessed plant size and number of leaves as measures of growth under current natural conditions and observed seed germination. I hypothesized that descendants are better adapted than the ancestors in their germination rate, plant performance, and plant survival under current conditions due to two decades of adaptive evolution to environmental changes. Finally, I discuss the conceptual integration of *in-situ* transplantations into the resurrection approach.

The aim of **Chapter II** was to investigate recent adaptive evolution of *Leontodon hispidus* to nitrogen and phosphorus enrichment and competition. After two intermediate generations, I grew ancestors and descendants in a common environment and applied a competition treatment using the natural competitor *Brachypodium pinnatum* (Poaceae). Furthermore, I applied nutrient treatments to plants that were subject to competition, supplying those plants weekly with either no nutrients, or with nitrogen (N), phosphorous (P), or both. I measured growth, leaf and floral traits, and I hypothesized that shifts in soil nutrient availability due to reductions of N emissions lead to a shift from aboveground competition for light to belowground competition for nutrients. Thus, I expected the evolution of lower competitive ability aboveground and higher competitive ability belowground. Further, I hypothesized that the decline of N emissions over recent decades selected for higher fitness in descendants of *L. hispidus* under low N availability. In contrast, I expected that descendants and ancestors respond similarly to high P availability due to a slower reduction of P emissions and greater persistence in the soils.

In **Chapter III**, I used the resurrection approach to investigate the effects of different experimental environments on the phenotypic differences between ancestors and descendants of *L. hispidus*. Furthermore, I wanted to test the effects of multiple intermediate generations on the phenotypic differences between ancestors and descendants. I grew two intermediate generations (F1 and F2) from ancestors and descendants under common conditions and cultivated them in three different experimental environments: in a garden, in a greenhouse, and in a climate chamber. I measured functional traits regarding important growth, leaf anatomy, and flowering phenology. I hypothesized (1) that conditions among experimental environments differ strongly, but phenotypic differences due to genetic differentiation should be detectable in every experimental environment. I expected (2), that phenotypic differences are

strongest in less controlled conditions, such as an outdoor garden, and are decreased in more optimal conditions, such as a climate chamber. Finally (3), I hypothesized that one intermediate generation might not be enough to sufficiently reduce non-genetic differences between ancestors and descendants due to the additional storage effects of ancestors, leading to lower performance of ancestors from F1 compared to ancestors from F2.

General Discussion

In the general discussion, I will start by highlighting the main findings of the three resurrection studies that I conducted. I will proceed to relate the results of **Chapter I** and **Chapter II** and discuss the results with regard to the evolution of the studied plant populations during the last three decades of environmental change. I will point out the implications of my work for conservation efforts with a focus on restoration projects using seed repositories. Then, I will discuss the reproducibility (**Chapter III**) of my results and evaluate the consistency of the results throughout all three chapters. Finally, I will discuss methodological caveats, present an outlook based on my findings, and give a general conclusion.

Main findings

In **Chapter I**, I transplanted seedlings and sowed seeds (only for *C. vulgare* and *L. hispidus*) from ancestors and descendants of my study species to their original sampling location in order to study adaptive evolution to the current climate. The study was performed in 2022, which proved to be an exceptionally hot and dry year in the study areas, and these extreme conditions led to a high mortality among study plants. Nonetheless, I detected evolutionary changes in all three study species, but the extent and direction of changes were species-specific. I found greater plant size and lower mortality in descendants of *M. ciliata* compared to their ancestors. These results can be explained by the evolution of higher drought tolerance or improved avoidance strategies. However, due to the missing intermediate generation, we cannot disentangle the effect of physiological adaptations of descendants or potential storage or maternal effects on the performance of the ancestors. Descendants of *C. vulgare* and *L. hispidus* tended to grow slower under field conditions than their ancestors, while especially descendants of *L. hispidus* strongly outperformed ancestors in the greenhouse before transplantation. Reductions in growth can be explained as maladaptation of the population due to inbreeding or genetic drift (Crespi, 2000) or as a response to the dry conditions as it lowers water loss by transpiration and reduces resource needs. In the first year, germination success for *L. hispidus* was very low due to the drought, but descendants still germinated faster than ancestors. Seeds of *C. vulgare* did not germinate at all in 2022, but in the following year, with more favorable

conditions, germination success was quite high, and descendants germinated with higher success than ancestors. The study populations might have evolved higher longevity in the dormant state (population of *C. vulgare*), leading to more viable seeds (Dalling et al., 2011). Alternatively, they might require less water for germination or have more efficient water uptake to facilitate germination (Baskin and Baskin, 2015). Overall, **Chapter I** demonstrates adaptive evolution of plant populations to the current environmental conditions and further highlights the importance of droughts as a strong driver of selection.

In **Chapter II,** I studied the evolution of competitive ability and responses to changing nutrient availability in natural plant populations over the last decades by conducting a resurrection study using ancestors collected 30 years ago and descendants collected in 2018 after growing two intermediate generations. I grew ancestors and descendants in a common environment and applied a competition treatment using the natural competitor *Brachypodium pinnatum*. Furthermore, I applied nutrient treatments to plants grown under competition and supplied them every week with either no fertilizer, or with nitrogen, or with phosphorus, or with both fertilizers. I found evidence the for evolution of increased competitive ability through faster growth belowground (root biomass), but also faster growth aboveground of descendants compared to ancestors. Hence, there was no shift in competitive ability from aboveground to belowground, as I expected. Furthermore, descendants had only higher competitive ability in treatments where N was absent (control and P-treatment), indicating that this population has evolved increased ability to compete for N. Nitrogen depositions decreased steadily over the last three decades, possibly driving competition and the observed evolutionary response. Finally, I observed the evolution of taller flower stems in descendants, which may be a strategy to compete better for pollinators, which may be driven by the existing pollinator decline in recent decades. The results of **Chapter II** show the complexity of underlying processes of contemporary evolution, including nutrient dynamics, competition, and potentially pollinators, and highlight the importance of understudied potential selection agents that can be investigated using resurrection studies.

In **Chapter III**, I assessed the effects of three different growth facilities (i.e., outdoor garden, greenhouse, and climate chamber) on the phenotypic differentiation

between ancestors and descendants of *L. hispidus*. I also evaluated differences in phenotypic expression between plants grown after one vs. two intermediate generations. I observed strong differences among plants growing in the different growth facilities. Descendants consistently had higher vegetative biomass and higher LDMC compared to ancestors in all three facilities. I also found a significant interaction between the growth facility and the temporal origin (ancestors vs. descendants): descendants had significantly larger rosettes than ancestors only in the greenhouse, and they flowered significantly later than ancestors exclusively in the climate chamber, which indicates that the evolution of delaying the onset of flowering may only be expressed under certain environmental conditions. I did not find significant differences between intermediate generations within the growth facilities, and thus, growing only one intermediate generation might be sufficient for the resurrection approach, at least for this species. Overall, this chapter demonstrates that the use of a particular growth facility and the chosen experimental conditions can dictate the presence and magnitude of phenotypic differences. This implies that absence of evidence is not evidence of absence when it comes to investigating genetically based trait differentiation among plant origins (in space or time). Therefore, experimental systems should be carefully designed to provide meaningful conditions, ideally mimicking the environmental conditions of the population's origins if the results should be translatable to natural conditions.

Rapid evolution of plant populations

All populations of the studied species were exposed to warmer temperatures, higher water deficits (droughts), and changes in nutrient availability over the last 25 years. I showed that all of these changes are potential selection agents that can shape the genetic structure of the plant populations. The effects of droughts have been widely studied in resurrection experiments because their effects can be very severe and exert strong selection pressure on populations in a short time and, thus, lead to rapid evolution. In **Chapter I**, I found compelling evidence for species-specific adaptations to drought in seed germination traits as well as in later life cycle stages. Descendants germinated faster and had more viable seeds than ancestors during and after a drought year, indicating evolution of increased longevity during dormancy of seeds. Little is known about how climate change is affecting germination (Cochrane, 2020), and seed

traits are largely understudied within resurrection studies, even though they are crucial for population persistence and can strongly influence the variability of the gene pool (Liu et al., 2022). It is likely that early life cycle stages are more susceptible to global change than adult life cycle stages and, therefore, warming and droughts will likely cause a decline in the number and diversity of establishing seedlings in the future (Cochrane et al., 2015; Walck et al., 2011). The results of **Chapter I** demonstrate the adaptive potential of seed traits in the case of two populations, underlining the importance of increasing the focus of resurrection studies on seed traits in order to understand the effect of climate change on germination and its adaptive potential on large scales.

Many resurrection studies on annuals report the evolution of drought escape strategies, i.e., accelerating the life cycle through faster growth and earlier onset of flowering to reproduce before water availability becomes limiting (e.g., Franks et al., 2007; Hamann et al., 2018; Lambrecht et al., 2020; Rauschkolb et al., 2023). I could not detect the evolution of drought escape strategies in the species I studied because *M. ciliata* did not initiate flowering during the experimental period. The descendants of *L. hispidus* and *C. vulgare* (**Chapter I**) did not grow faster in the field to escape the drought, and they died before I was able to record their flowering phenology. Under certain controlled conditions, descendants even flowered later than their ancestors (*L. hispidus,* **Chapters II** and **III**). Reduced growth was found in the same populations by Rauschkolb and colleagues (2022a) and can be either explained as maladaptation through gene drift or inbreeding (Crespi, 2000) or as a strategy to reduce water loss through decreased transpiration and lower resource requirements (Basu et al., 2016). Even though descendants of *L. hispidus* grew slower in the dry field conditions, they grew faster than their ancestor under more benign greenhouse conditions with regular watering before the transplantation. A similar result can be observed in the other two chapters. In **Chapter II** and **III**, descendants of *L. hispidus* generally outperformed ancestors in growth with either more vegetative biomass or larger rosette diameter. Thus, descendants appear to be able to use resources more effectively when they are abundant. With increasing variability and decreasing predictability of environmental conditions, effective resource use in a favorable period can be a good strategy to establish quickly and consequently increase survival chances when more stressful conditions occur. This strategy might be connected with the observed delay in flowering

in the very controlled and stable conditions of the climate chamber in **Chapter III** by prioritizing vegetative growth over early flowering. However, this hypothesis must be further investigated by applying treatments to ancestors and descendants that provide the plants with different variability and predictability of resources (March-Salas et al., 2019).

Reproductive traits such as the onset of flowering are very important to consider in evolutionary studies because they can influence, and be themselves influenced by, several ecological and evolutionary processes, such as mating patterns, gene flow, and interactions with animal pollinators (Franks, 2015). The onset of flowering depends on environmental cues, and plants can accelerate it or delay it in order to optimize seed production and dispersal conditions for the next generation (Coupland, 1995). In a similar study on the same population of *L. hispidus*, Rauschkolb and colleagues (2022b) also observed evolution of delayed flowering and explained it with the introduction of sheep grazing in 2007, which pressured plants to flower later to escape the grazing. A delay in flowering has also been observed as a result of winter and spring droughts and correlates with dehydration avoidance strategies (Kooyers, 2015; Melgar et al., 2012; Monroe et al., 2018). Therefore, it is also possible that, apart from grazing pressure, droughts selected for later flowering plants in *L. hispidus*.

Increasing temperatures and changes in precipitation patterns may also affect plant populations by increasing competition for water, which can be especially strong in already dry habitats such as calcareous grasslands. Additionally, these habitats are usually limited by nitrogen (Maskell et al., 2010), and since nitrogen availability is regulated by soil moisture (Taiz and Zeiger, 2006), water stress can also indirectly increase competition for this specific nutrient. Soil microbial processes are also extremely dependent on soil moisture, such as litter decomposition into plant-available nutrients or denitrification (Brady and Weil, 2002). A topography with many slopes, which is the case for the locality of the studied populations, may further reduce water availability. Additionally, the slope may increase runoff of nutrients and wash them out before they can accumulate in meaningful quantities (Li et al., 2006). Together with the decreasing N emissions, all these factors may actually limit nutrient enrichment. In line with assumptions, nutrient analyses of soil samples (**Chapter II**) showed that N content was comparable to other calcareous grasslands, and P content was even comparably

low. Competition for these resources is an important factor to consider for evolutionary responses (Craine and Dybzinski, 2013) and may be especially important for the populations I studied because the increasing dominance of the grass *Brachypodium pinnatum* threatens biodiversity in their habitat (Bąba, 2003; Bobbink and Willems, 1987; Canals et al., 2017). In general, **Chapter II** revealed the evolution of higher competitive ability in the population of *L. hispidus* belowground for water and nutrients as well as aboveground for light and space against *B. pinnatum*. The belowground competitive ability is characterized mainly by faster growth of the roots, which can be beneficial to reach deeper water deposits under water stress, but also expedites nutrient uptake (Freschet et al., 2021a). However, I only measured root biomass, whereas root ecology is much more complex, with an array of root traits that can provide more detailed insights into nutrient and water uptake, as well as regenerative capacity during droughts. These traits include the morphology of the (absorptive) root, the presence and size of tap roots, or rhizomes (Freschet et al., 2021b). Nonetheless, the selection for this evolutionary response in root biomass may be either facilitated directly by the increased competition with roots of the dominant *B. pinnatum*, by lower water availability, by the changes in nutrient dynamics or a combination of these factors. Compared to consecutive drought years, the selection pressure of the gradual changes in nutrient availability may be much slower. Furthermore, the impact of slowly reducing N emissions may be small since the habitats of the studied populations may not have accumulated high quantities of nutrients in the past due to the topography, and the populations may already be adapted to low nutrient conditions. However, due to the lack of data on soil nutrients in the past, it is not possible to make precise statements on the nutrient dynamics of the recent years in the studied population of *L. hispidus*.

In general, my results show the occurrence of rapid evolution in three plant populations from different species in response to recent environmental changes. While my results demonstrate the capacity for rapid evolution, it is still unclear whether the evolved traits provide evolutionary rescue and whether the pace of evolution can keep up with rapid environmental changes in the future. While the common-environment approach allows the demonstration of evolutionary trait shifts, it is not possible to disentangle different selection agents, as they cannot be directly measured. Moreover, non-directional evolutionary processes (genetic drift, gene flow) cannot be ruled out

without accounting for random trait variation. To verify that the observed evolution (i.e., shift in allele frequencies) was caused by selection, the resurrection approach can be combined with genomic tools such as Q_{ST}-F_{ST}-analyses. Here, the divergence of quantitative traits (Q_{ST}) is compared with the divergence of neutral genetic molecular markers (F_{ST}), and this comparison makes it possible to validate whether trait divergence has risen through natural selection (Leinonen et al., 2013). This method has already revealed extensive genomic heterogeneity and adaptive differentiation in many taxa and traits, primarily among different populations. Q_{ST}-F_{ST}-analyses can also be performed on ancestors and descendants of the same population, which Rauschkolb and colleagues (2022b) demonstrated on the same population of *L. hispidus* that was studied in my experiments and found strong evidence for directional selection over time in flowering traits. Artificial selection experiments (i.e., experimental evolution) can also be implemented within the resurrection approach to assess the evolutionary potential of descendants in response to future selection pressures and to confirm the impact of past selection pressures (Franks et al., 2018a; Johnson et al., 2022). Ancestors and descendants are compared under common conditions and exposed to a certain selection pressure (e.g., low water availability) for multiple generations. With this method, it can be shown if and how fast ancestors can reach the genotype of descendants through selection, providing more proof of the underlying processes of population evolution.

Implications for restoration

Seed repositories can be a great source of seed material for resurrection studies, but they are also extremely useful for the conservation and restoration of plant populations in this era of biodiversity loss (Liu et al., 2018). By 2030, the Global Biodiversity Framework aims to restore 30% of degraded habitats, and in order to reach that goal, seed repositories will play a very important part in providing the needed seed material (CBD, 2022). They can provide seeds sampled from wild populations, which is especially valuable for the reintroduction and recovery of threatened or already extinct species or local populations (Wambugu et al., 2023). It is often argued that locally sampled seeds should be used for restoration, as they should be adapted to the local climate, soil and biotic interactions (Kiehl et al., 2014). However, there is concern that those seeds might have lost their local adaptation due to the rapid environmental

changes, especially if the seed material has been stored for an extended period of time (Prober et al., 2015). Instead, it is proposed to use seed material from populations that already experience climatic conditions similar to the climatic scenario predicted in the future for a given restoration location (Crowe and Parker, 2008; Sgrò et al., 2011). However, this approach disregards the local adaptations to soil and biotic interactions, which might not change along with climate change (Bucharova et al., 2019).

In **Chapter I**, the use of seed material from a seed repository and their reintroduction to the collection sites in nature reserves have direct implications for restoration ecology. The transplantations of juvenile plants revealed that descendants of *M. ciliata* were better adapted to the natural conditions and established better than their ancestors. For the other two species, the transplantations failed, as both ancestors and descendants reached 100% mortality without detectable differences. However, in **Chapters II** and **III**, descendants of *L. hispidus* generally had higher competitive ability and performed better than the ancestors under controlled conditions, which suggest that there could have been differences in the field experiment as well, had the temporal resolution of repeated measurements been higher and/or had environmental conditions of the transplantation year not been that extreme. Therefore, it is important to carefully choose the timing of transplantations for restorations to make sure that the conditions favor the establishment of young seedlings. Additionally, closely monitoring the environmental conditions may be necessary, and intervention in case of extreme conditions might be needed (e.g., with watering). For restoration via seed sowing, the results of **Chapter I** also suggest a higher adaptive capacity in seed traits of descendant seeds as they germinated faster and had higher viability through dormancy. Generalizations should be made with care since I only investigated one population per species, but the results suggest that the populations have adaptive potential and did not completely lose their local adaptation. Therefore, I recommend not using seeds for restoration that have been stored for an extended time period and instead using contemporary seeds, if available. My results can only provide insight into the evolutionary processes of the last three decades, but inferring the population's adaptive potential for future climate change is a challenging task. Adaptive potential is directly connected to high genetic diversity to promote the selection of the best-suited genotypes (Breed et al., 2013). Therefore, Bucharova and colleagues (2019) recommend the use of seed mixtures sampled from multiple

populations around the target location, which provides a compelling compromise between using locally adapted seed material and providing sufficient levels of high genetic diversity to ensure adaptive potential in the future. Ancestral seed material should still be conserved because it is very useful for resurrection and germination studies, and it may contain valuable alleles that have been lost in the wild population. Based on the insights generated in this thesis, restoration efforts could be combined with the resurrection approach by reintroducing ancestors next to descendants to provide valuable insight into the evolution of more populations of potentially threatened species.

Reproducibility and methodological limits

To carry out resurrection studies under common-environment conditions, researchers can use various growth facilities such as the outdoor garden, greenhouse, or climate chamber. These different approaches can differ immensely in their environmental conditions, such as light and water availability, temperatures, heterogeneity, and the variability in these factors (Poorter et al., 2012). Therefore, it is likely that different growth facilities cause plants to express different phenotypes due to phenotypic plasticity (Sultan, 2000). If we compare genotypes with significant genetic differentiation (e.g., ancestors and descendants), we may expect that the comparisons would show qualitatively similar results across facilities. However, this assumption has not been tested thoroughly in the framework of common-environment experiments. Most studies only use a single growth facility, and it is unclear if the results can be compared with the results of another study performed in a different growth facility. There is a possibility that phenotypic differences can only be observed under certain environmental conditions or could even show contrasting results depending on the environment. Therefore, incomplete conclusions could be drawn from an experiment that is only conducted in a growth facility.

Table 2. Comparisons of trait responses of *L. hispidus* throughout all three chapters and a study by Rauschkolb and colleagues (2022b) on the same population. Considered are only the control groups of the experiment. A significantly higher value of descendants compared to ancestors is indicated with a plus sign (green). No significant differences between ancestors and descendants are indicated as ns (grey) and not measured traits are indicated with no data (white). Plants from **Chapter II** were in the greenhouse for four weeks before they were moved to an outside garden.

	Chapter I	**Chapter II**		**Chapter III**		**Rauschkolb et al. 2022b**
Trait	Greenhouse	Greenhouse/ Garden	Garden	Greenhouse	Climate chamber	Greenhouse (Tübingen)
Rosette diameter	+	+	ns	+	ns	+
Vegetative biomass	no data	ns	+	+	+	ns
LDMC	no data	no data	+	+	+	+
Onset of flowering	no data	ns	ns	ns	+	+
Reproductive biomass	no data	ns	ns	ns	ns	ns
Number of flowers	no data	ns	no data	no data	no data	ns

All three of my chapters include seed material from the same population of *L. hispidus*, which enables me to assess the robustness or reproducibility of my results (Table 2). Additionally, Rauschkolb and colleagues (2022b) studied the same population and seed material in a greenhouse in Tübingen using the resurrection approach, which can also be related to my results (Table 2). There are some key differences among these studies that need to be considered before comparing the results. Rauschkolb and colleagues (2022b) used seeds from five seed families of each temporal origin after one intermediate generation, and they stratified the seeds at 5°C in the dark for one week prior to their experiment. For my studies in **Chapters I** and **II**, I used seeds from a second intermediate generation since the seed material from the first intermediate generation was limited. This allowed me to use more seed families for my experiments (12 seed families for **Chapter I** and nine seed families for **Chapter II**) because I could propagate the seed families that could not be used in the study by Rauschkolb and colleagues (2022b) due to insufficient amount of seeds. In **Chapter III**, I used seeds from the first and the second intermediate generation, which offers the possibility to make comparisons among the studies. Finally, I did not stratify the seeds of my experiments, because it was not required to promote fast germination.

Overall, I did not find any contrasting results throughout all experiments, but indeed, not all differences in traits between ancestors and descendants were consistent. Only the measurements of leaf dry matter content (LDMC), reproductive biomass, and number of flowers were consistent throughout all experiments. Descendants always had a higher LDMC and did not differ in reproductive biomass and number of flowers from ancestors. Therefore, there is mounting evidence that LDMC has evolved in this population of *L. hispidus* and might be expressed irrespective of the environment. High LDMC can help to decrease leaf evapotranspiration as a strategy to reduce water stress and could have been selected in this population by increasing temperatures and heatwaves caused by climate change (IPCC, 2018). For rosette diameter, I observed that phenotypic differences between ancestors and descendants depended on the experimental environment. Descendants had a larger rosette diameter than ancestors in all studies using a greenhouse (**Chapter I**, **Chapter III**, and in Tübingen) and in **Chapter II**, where the plants were first in the greenhouse for four weeks and were then moved to an outside garden. There were no significant differences between temporal origins in rosette diameter in the outside garden and in the climate chamber of **Chapter III**. As discussed in **Chapter III**, the most prominent distinction of the greenhouse compared to the other experimental environments is the low light irradiance. Increasing rosette diameter may be a good strategy to increase the surface area of the leaves to capture more light. An explanation for the evolution of this strategy could be the evolution of a plastic response to shading, which could have been selected by the competition with the dominant grass *Brachypodium pinnatum* (Bąba, 2003; Bobbink and Willems, 1987) as it can substantially shade the rosette of *L. hispidus*. Interestingly, descendants had higher vegetative biomass than ancestors in all experimental environments of **Chapter III** but not in **Chapter II** and the study by Rauschkolb et al. (2022b). The difference could be either due to different experimental designs, since the plants in **Chapter II** were in the greenhouse the first four weeks and then moved outside, or the vastly different conditions in the greenhouse in Tübingen compared to the greenhouse I used (e.g., different lamps, different amount of shading, heating, different watering or fertilizer). Furthermore, the season in which the experiments were conducted could affect the results since the seasons can greatly affect outdoor garden and greenhouse conditions (Poorter et al., 2016). Although the data cannot pinpoint the exact cause of the discrepancies among the studies, it illustrates well that not only different experimental

approaches can produce contrasting results, but there can also be great variance among similar experimental approaches.

My results suggest that the experimental environment can greatly affect the expression of phenotypic differences among genotypes and, consequently, bias conclusions. Comparisons of common-environment experiments with other studies should made with care and under consideration of the differences in experimental conditions. Furthermore, experimental conditions should be chosen with great care to allow optimal conclusions regarding the research questions. Greenhouses are the most used experimental environment in resurrection studies (e.g., Anstett et al., 2021; Franks et al., 2007; Gay et al., 2022; Hamann et al., 2018; Kuester et al., 2016; Lambrecht et al., 2020; Nevo et al., 2012; O'Hara et al., 2021; Sultan et al., 2013; Vtipil & Sheth, 2020), but greenhouses provided an experimental environment with the least representative natural conditions and have some drawbacks such as shading and peaking temperatures (Poorter et al., 2012). For example, when investigating adaptive evolution, *in-situ* experiments would be a good option, as plants are studied in their natural environment, where they are expected to have evolved. The plants will have access to their natural soil, competitors, and pollinators. Naturally, *in-situ* experiments are often associated with a higher risk of failure due to transplantation shock, extreme weather conditions, or herbivory, and it is often difficult to disentangle the factors the plants are responding to. However, the valuable insights gained from *in-situ* experiments will be worth the risk, and careful planning with close monitoring of the environmental conditions will further help to increase the chances of success. Researchers could even combine *in-situ* designs with experimental treatments to simulate expected future selection pressures and investigate the evolutionary potential of plant populations. Ideas include rainout shelters to simulate even drier conditions, different levels of mowing to test for competition responses and watering treatments to simulate extreme precipitation events. Outdoor garden experiments are also a good, less risky option to study rapid evolution due to the more natural conditions and contemporary climate. Finally, using climate chambers can prove to be a valuable approach, especially if the natural environmental conditions of the study populations are known. Researchers can realistically program climate chambers to simulate the natural environment, simulate their environmental conditions of the future, and apply

treatments. Further controlling and simulating plant-soil and plant-plant interactions will be especially valuable to strengthen the explanatory power of the results.

There are some limitations to my approach that could affect the robustness of the results. First, the production of an intermediate generation failed for *M. ciliata*, which might compromise the validity of results because the seed quality of the ancestral seeds might be affected by the storage conditions and storage time or influenced by parental effects (Franks et al., 2018). Another confounding factor for the interpretation of the results can be the so-called “invisible fraction”, i.e., mortality during storage might genetically correlate with post-emergence plant traits (Weis, 2018). However, since the germination rate of the ancestral seeds was very 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.* The bias on *C. vulgare* might be limited as well, given that the fresh descendants seeds had a similar germination rate (40%). Furthermore, I only studied one population per species, and **Chapters II** and **III** only focus on one population of *L. hispidus*, which is also hampering generalizations, as adaptive potential can vary greatly among populations of the same species (Franks et al., 2007; Wooliver et al., 2020). I only compared the ancestral genotypes to one descendant sample with a long time span in between (26 – 28 years), which makes it hard to pinpoint the selection agents that caused the genetic differentiation among the sampling years. It is often assumed that evolutionary changes build up gradually over time, but these could also be the result of extreme selection events in single years (Fig. 5; Gould and Eldredge, 1977). Hamann and colleagues (2018) applied the resurrection approach to a population with regular samplings across two decades and showed that evolution can be very dynamic and can highly depend on a given year (e.g., wet vs. dry year). Therefore, it is advisable to use consecutive years of sampling in order to gauge whether the evolutionary response is steady and directional or fluctuates strongly with climate variability.

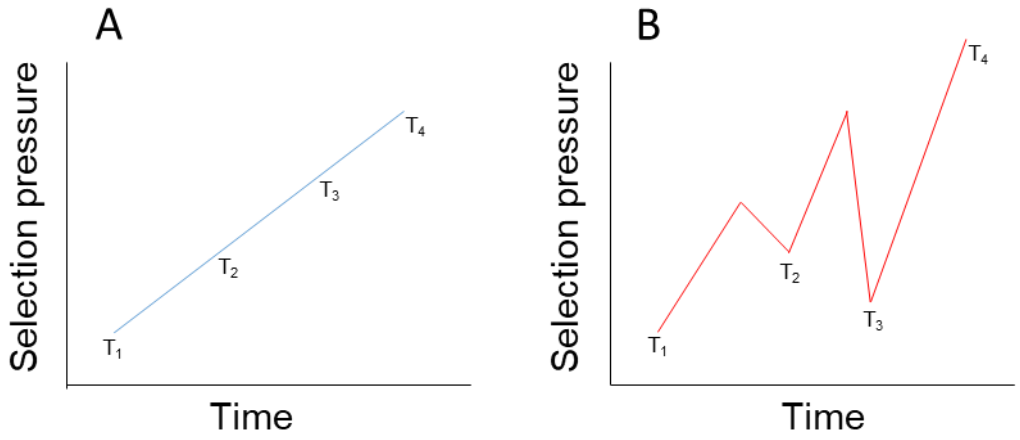

Figure 5. Different forms of selection pressure over time. Increasing selection pressure can be applied to plant population gradually and linear over time (A) or in a more dynamic way (B) due to environmental variability. Shown are exemplary sampling times for a given plant population (T_1 – T_4), demonstrating the benefit of using multiple sampling times to increase the detectable resolution of potential selection pressures on a plant population.

Using seed repositories for the ancestral samples can have some drawbacks, as collected seeds are often bulked, and it is not known how many mother plants have been sampled. Furthermore, I only used 15 random individuals from the seed bulks for the intermediate generation, which introduces some uncertainty about the distribution of seed families and about the possibility of genetic bottleneck. However, the ancestral seed material appears to be comparable to descendant seed material in its genetic structure, and both samplings represent the genetic diversity of the populations because the genomic analysis showed similar relatedness of plants between temporal origins and similar allelic richness (Rauschkolb et al., 2022a). Finally, the number of seed families used in the experiments was sometimes not very high (e.g., nine seed families in **Chapter II**) because not all mother plants from the intermediate generation produced sufficient seed material. It is imaginable that the low amount of seed families does not represent the full genetic diversity of the study population and there might have been artificial selection during the intermediate generation because six of the 15 seed families were completely disregarded. However, since we randomly pollinated each of the 15 plants with each other during the intermediate generation, the genetic diversity of all 15 plants should be present in every seed family to some extent and would again reduce this bias.

Outlook and next steps for the resurrection approach

The resurrection approach is becoming more widely used to determine rapid evolution of plant populations, and this method is very promising to advance our understanding of rapid plant adaptations in this age of global change. Based on the findings of the studies conducted in this thesis, I am able to provide useful recommendations for the next steps of the resurrection approach (Fig. 6). Ancestral material already stored in seed repositories provides immediate opportunities in the future to conduct resurrection studies. Dedicated large-scale seed repositories for research purposes, such as 'Project Baseline' (www.baselineseedbank.org) will provide further opportunities while ensuring proper sampling protocols and consistent methodological procedures (Etterson et al., 2016). Sampling multiple populations over consecutive years will improve the robustness of the results and make generalizations more approachable. Furthermore, combining the resurrection approach with other methods will be valuable to offset some limitations of resurrection studies. For instance, its combination with transplant studies will provide firm proof of adaptive evolution and should be carried out more extensively in future studies. Applying Q_{ST}-F_{ST} analyses to ancestor and descendant pools and other molecular and genetic tools can contribute to disentangle the possible underlying processes that can cause evolution, such as selection, genetic drift, and mutation. Resurrection studies would also benefit from focusing more on germination traits, which can have strong impacts on the genetic structure of plant populations and plant fitness.

It is also important to consider the implications of plant-microbial interactions for adaptive potential, as many plants form positive associations with fungi, bacteria, and viruses, which can strongly influence plant fitness and physiology (Hamann et al., 2021). These microbial organisms can be strongly affected by drought events and resource availability but have also been shown to contribute to drought tolerance and nutrient stress in plants (Kim et al., 2012). Therefore, considering plant-microbe interactions in evolutionary studies will be valuable to gain deeper understanding of adaptive processes. Performing transplantation experiments in the original habitat, as was done in **Chapter I**, is a good way to integrate plant-microbe-interactions, because plants will have access to their natural microbiome. It would also be feasible to sample local soil of the population when seeds are sampled for the ancestral (if possible) and

descendant seed material, respectively. This soil can then be used to create full-factorial treatments and inoculate sterilized soil in common-environment experiments to detect changes in soil biota and test for plant adaptations to these changes if they occurred. However, care has to be taken as long storage of soil samples may affect the viability of soil biota (Birnbaum et al., 2017).

When applying treatments to determine potential drivers of rapid evolution, exploring understudied drivers such as soil nutrients, competition, and pollinators would be especially valuable to gain further insight into the complexity of selection agents. Rapid global change involves plenty of changing factors, and implementing multiple treatments in resurrection studies in full factorial designs, rather than investigating them one by one, can greatly improve our understanding of the underlying processes of rapid plant adaptations.

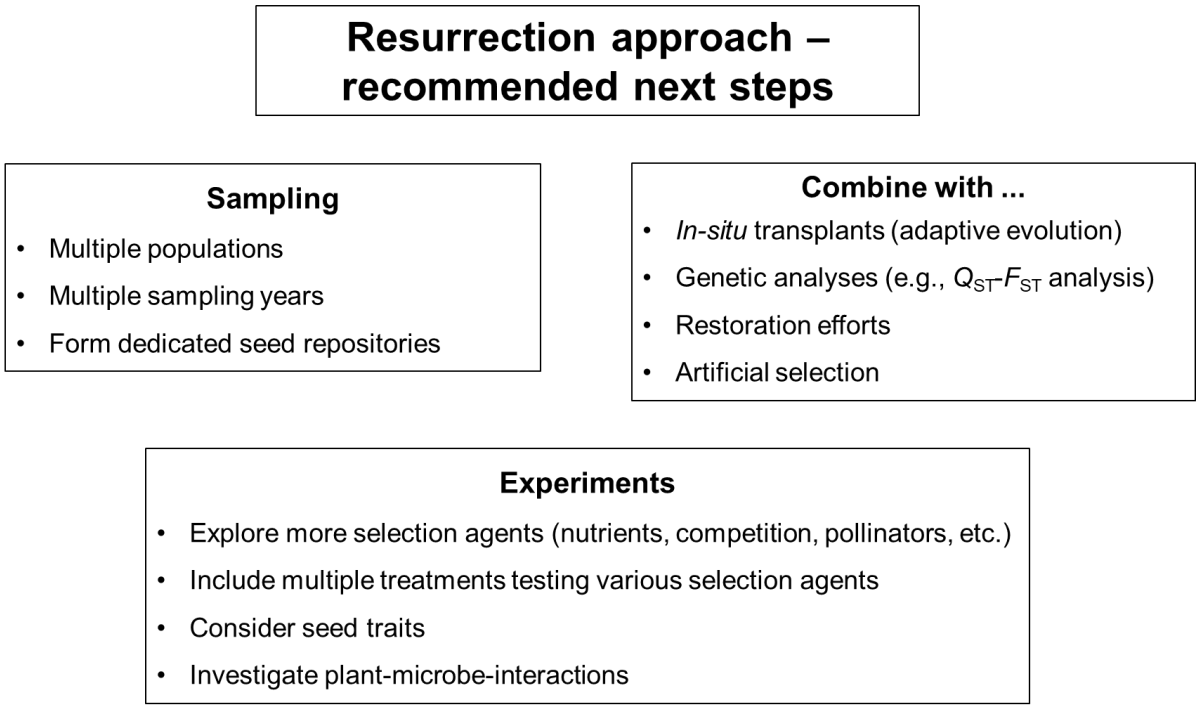

Figure 6. Recommendations for applying the resurrection approach regarding sampling methods, implementing other experimental approaches, and underexplored research aims in experiments.

Conclusions

In this thesis, I presented a series of experimental studies that have made important contributions to the field of resurrection ecology, in particular, and evolutionary biology in general. Overall, my work demonstrated rapid evolution of multiple plant populations and provided the first steps in new directions for the resurrection approach. My thesis exemplified the incorporation of *in-situ* transplantations into the resurrection approach as an important step to infer adaptive evolution. In future resurrection studies, the combination with *in-situ* transplantations will be valuable to confirm whether observed evolutionary shifts in common-environment experiments translate to improved plant performance in their natural environments. Furthermore, my results indicated the implications of the complexity of contemporary evolution and shed light on the importance of studying a wide array of selection agents. Finally, I provided considerations for the design of common-environment experiments to ensure robust interpretations. Despite some methodological limitations, future studies can greatly benefit from the insights generated in the presented studies and can continue to strengthen our understanding of rapid plant evolution to the complex selection pressures that plant populations face in this era of global change.

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Annex

Chapter I

Declaration of author contributions to the manuscript: Combining the resurrection approach with transplant experiments to investigate adaptation of plant populations to environmental change

Status: accepted in *Perspectives in Plant Ecology, Evolution and Systematics* on the 29.11.2023

Contributing Authors: P. Karitter, M. March-Salas, A. Ensslin, R. Rauschkolb, S. Godefroid, J.F. Scheepens

What are the contributions of the doctoral candidate and the co-authors?

1. Concept and design
The experimental concept was developed by the doctoral candidate (20%), AE (10%), RR (15%), SG (10%) and JFS (45%). The experimental design was planned by the doctoral candidate (33%), SG (33%), and JFS (33%).
2. Conducting tests and experiments
Experimental setup and measurements were performed by the doctoral candidate (45%) and SG (45%) and JFS (10%).
3. Compilation of data sets and figures
Data were compiled and illustrations prepared by the doctoral candidate (90%). One Figure is based on an illustration by JFS (10%).
4. Analyses and interpretation of data
Data were statistically analysed by the doctoral candidate (60%), MMS (20%), and JFS (20%). Data interpretation was performed by the doctoral candidate (50%), MMS (15%), AE (5%), RR (5%), SG (5%), and JFS (20%).
5. Drafting of manuscript
The first draft of the manuscript and all subsequent revisions were made by the doctoral candidate (50%). Additional contribution and comments were made by MMS (15%), AE (5%), RR (5%), SG (5%), and JFS (20%).

I hereby certify that the information above is correct.

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Research article

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

Pascal Karitter [a,*], Martí March-Salas[a], Andreas Ensslin[b], Robert Rauschkolb[c,d], Sandrine Godefroid [e], J.F. Scheepens[a]

[a] Plant Evolutionary Ecology, Institute of Ecology, Evolution and Diversity, Faculty of Biological Sciences,GoetheUniversity Frankfurt, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany
[b] Conservatory and Botanic Garden of the City of Geneva, Chemin de l'Impératrice 16 1, 1296 Chambésy, Geneva, Switzerland
[c] Institute of Ecology and Evolution with Herbarium Haussknechtand Botanical Garden, Department of Plant Biodiversity, Friedrich Schiller University Jena, Germany
[d] German Centre for Integrative Biodiversity Research(iDiv) Halle-Jena-Leipzig, Leipzig, Germany
[e] Meise Botanic Garden, Nieuwelaan 38, 1860 Meise, Belgium

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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 seedsfrom the samepopulation. 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 seedlingsfrom 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 seedsof 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 and larger plant size 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. Descendantsof *C. vulgare*seedlings tended to be smaller and descendants of *L. hispidus* seedlings produced fewer 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 seedsof *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 conditionsare rapidly changing sincethe last decades and will continue to changein the future (IPCC,2018). In North-western Europe, precipitation is expectedto decrease,especially in summer, and evapotranspiration will increasedue 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). Thisapproach consistsof an experimental design that usesseedsfrom a population collected before (ancestors) and after (descendants) a potential selection pressure (e.g., consecutive years of drought, changesin nutrient supply, etc.). Comparing phenotypes of these two generations in a common environment may then

* Corresponding author.
*E-mail address:*karitter@bio.uni-frankfurt.de (P. Karitter).

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reveal evolutionary changes (Franks et al., 2007). Recently, seeds collected for storage in seedrepositories 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 changes in responseto drought stress(Franks et al., 2007; Nevo et al., 2012; Sekor and Franks,2018; Thomann et al., 2015; Vigouroux et al., 2011). Many resurrection studies using predominantly annual speciesreported evolution of earlier onsetof flowering after a few yearsof intensedroughts (Franks et al., 2007; Nevo et al., 2012; Thomann et al., 2015). In some species,seedsalso 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 escapestrategyinvolving rapid developmentto complete the life cycle while resourcesare 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 and Sheth,2020; Wooliver et al., 2020; Rauschkolbet 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, air temperature, and water and nutrient availability are often much more benign under controlled conditionsand plantsusually do not experience realistic winter conditions (Poorter et al., 2016). Moreover, temporal dynamicsof 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 under field conditions (Kawecki and Ebert, 2004). To convincingly demonstrate local adaptation, reciprocal transplant experiments remain the gold standard (Kawecki and Ebert, 2004; Blanquart et al., 2013). Consequently, resurrection studies should be combined with in situ transplantations in the sites of population origins (Franks et al., 2018), but to our knowledge no such study has 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 and Ebert, 2004; Fig. 1). This method can be partially applied to the resurrection approach by transplanting genotypesin time instead of in space. Naturally, descendantscannot be transplanted to the past, but ancestors and descendantscan be transplanted to the exact location where they were sampled in the present(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 seedbank material, from ex situ cultivation and from a sampledwild population closeto the original population. However, their study did not focus on investigating evolutionary change, but rather the effect of ex situ cultivation on germination and establishmentof plants reintroduced into the wild.

Here, we investigated recent adaptive evolution of three plant species from calcareous grasslandsin Belgium by conducting a resurrection study in combination with in situ transplantations. We used ancestors sampled 23–26 yearsago and contemporary descendantsof a population of *Melica ciliata, Clinopodium vulgare* and *Leontodon hispidus*. We transplanted seedlings and sowed seedsfrom ancestors and descendants to the original sampling location. We measured mortality as an inherent component of individual fitness and direct indicator of selection pressure in plant populations(Violle et al., 2007). We also assessedplantsize and number of leaves as measures of growth under current natural conditions, and we observed germination of the seeds.We hypothesized that descendantsshow stronger adaptation than their ancestorsin terms of higher germination rate, better plant performance and lower plant mortality under current conditions due to two decadesof adaptation to environmental changes. Finally, we discussthe 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 of 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 speciesare 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).

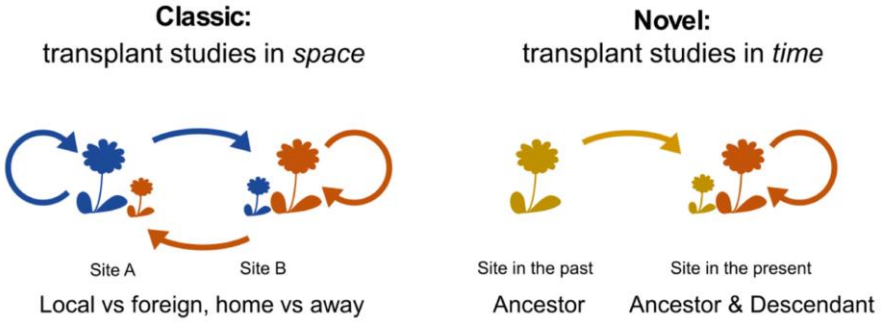

Fig. 1. Comparison of the classical approach of transplant experiments in space (left) with the novel approach of transplant experiments in time (right). Classical reciprocal transplantation experiments aim to measure local adaptation and fitness trade-offs by transplanting plants from different populations 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 processesthrough time.

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 1.1 km for *M. ciliata*, 4.1 km for *C. vulgare*, and 1.9 km for *L. hispidus*. Seed material of the ancestors was 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 results in an upper limit of generations between temporal origins of 23 generations for *C. vulgare*, 26 generations for *L. hispidus* and 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. They 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 to 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 was 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. S2B). 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

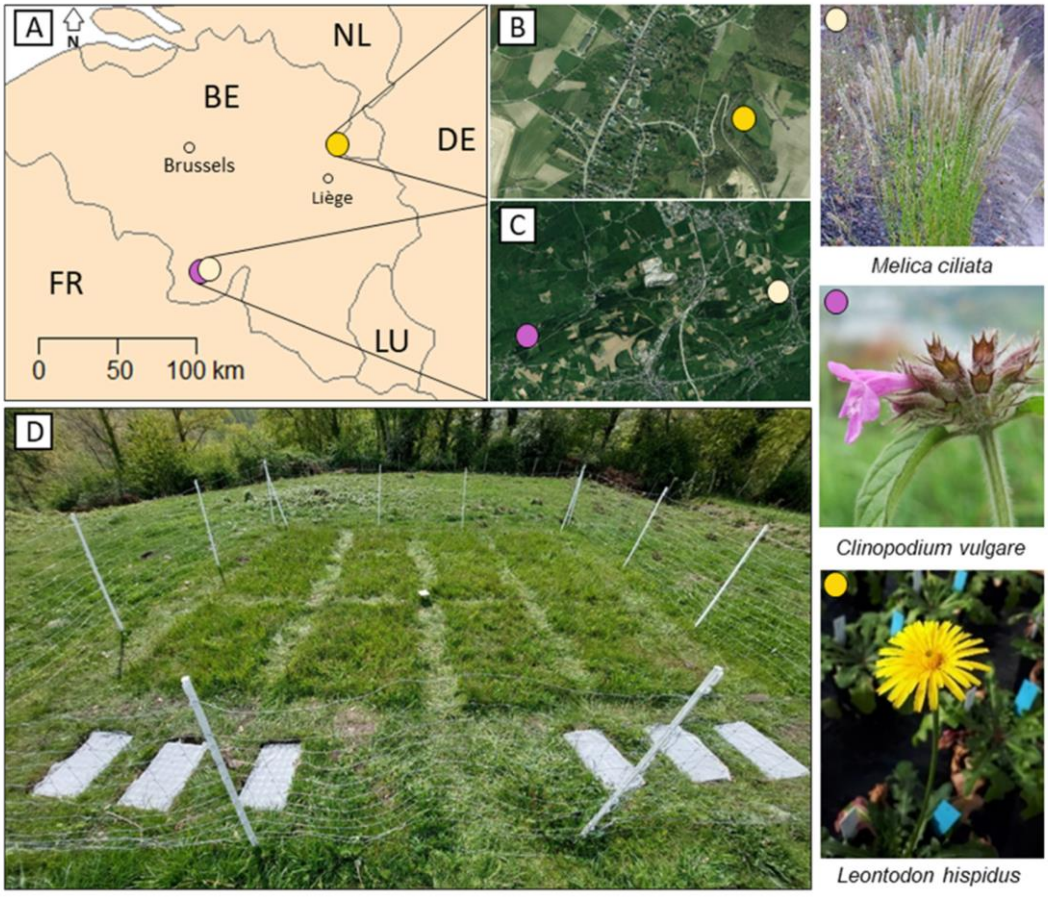

Fig. 2. Map of the focal populations in nature reserves in Belgium. Overview map of Belgium (A) and zoomed-in areas of the focal populations (B & C). Photograph of an exemplary experimental site (D) with six multitray-pots that contained the sowed seeds and are covered with nets. The populations of the 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.

(1994). The temperature anomaly steadily increased from 1966 until 2020 and mean temperature in the seedcollection years was 9.41 °C in 1992 (ancestors) and 10.25 °C in 2018 (descendants; Fig. 2SB). The average CWD steadily increased during the period from 1966 to 2020 and was 66% higher in 2018 compared to 1992 (Fig. 2SA). The focal populations were thus exposed to a progressively diminishing water availability in responseto 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 seedsof each temporal origin and 15 seedlings were randomly selected and cultivated for eachtemporal origin. We kept the plantsfrom 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 a second refresher generation was grown using the newly produced seedswhile maintaining separation of temporal origins, but this time bumblebees (Natupol Seeds, Koppert GmbH) were used for pollination. The refresher generation failed for *M. ciliata*, becausethe plants did not flower, so for this species we used the ancestral and descendant seed material without a refresher generation in our experiments.

In September 2021, refreshed seedsfrom 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. We deemed the selected plots to be suitable for the experiment, since we took care to place them in the same habitat with the same topography. To prevent herbivory and trampling by large grazers, each plot was fenced. We cut the standing vegetation with a brush cutter to facilitate transplantations. The plots were divided into 12 blocks for *C. vulgare* and *L. hispidus* and into 10 blocks for *M. ciliata* (Fig. 2D). 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 seedfamily, but randomized within each block. In February 2022, we visited the plots to sow seeds in local soil 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 multitray-pots (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 (Fig. 2D).

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 blocksto measure soil temperature every 4 h. 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 0.3–1 g of sieved soil was milled with the Mixer Mill MM400 (Retsch, Haan, Germany) for 60 swith 30 roundsper 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 analysed 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. Measurementsin the field

The plots were revisited in April 2022 and we measured the following: germination, mortality, number of leavesand 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 analyses 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 seedfamilies), we created a completely random subset of the descendantsconsisting 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 (Bateset al., 2015) to analyse 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 seedfamily (for data of *C. vulgare* and *L. hispidus*), block and individual (to account for multiple measurementsover 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 analysed whether died plants had a different plant size from plants that survived in the previous measurement using LMMs. Therefore, 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 responsevariable 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 analyse 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 pot as random factors. We checked the model for zero inflation using the function "check_zeroinflation" from the *performance* package (Lüdecke et al., 2021). The Poisson 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. We calculated marginal R^2 (R^2_m) and conditional R^2 (R^2_c) for these LMM models using the "r2" function of the *performance* package (Lüdecke et al., 2021). For all GLMMs, we calculated pseudo-R^2_m and pseudo-R^2_c using the "r.squareGLMM" function with the delta method from the *MuMIn* package (Bartoń, 2023). 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

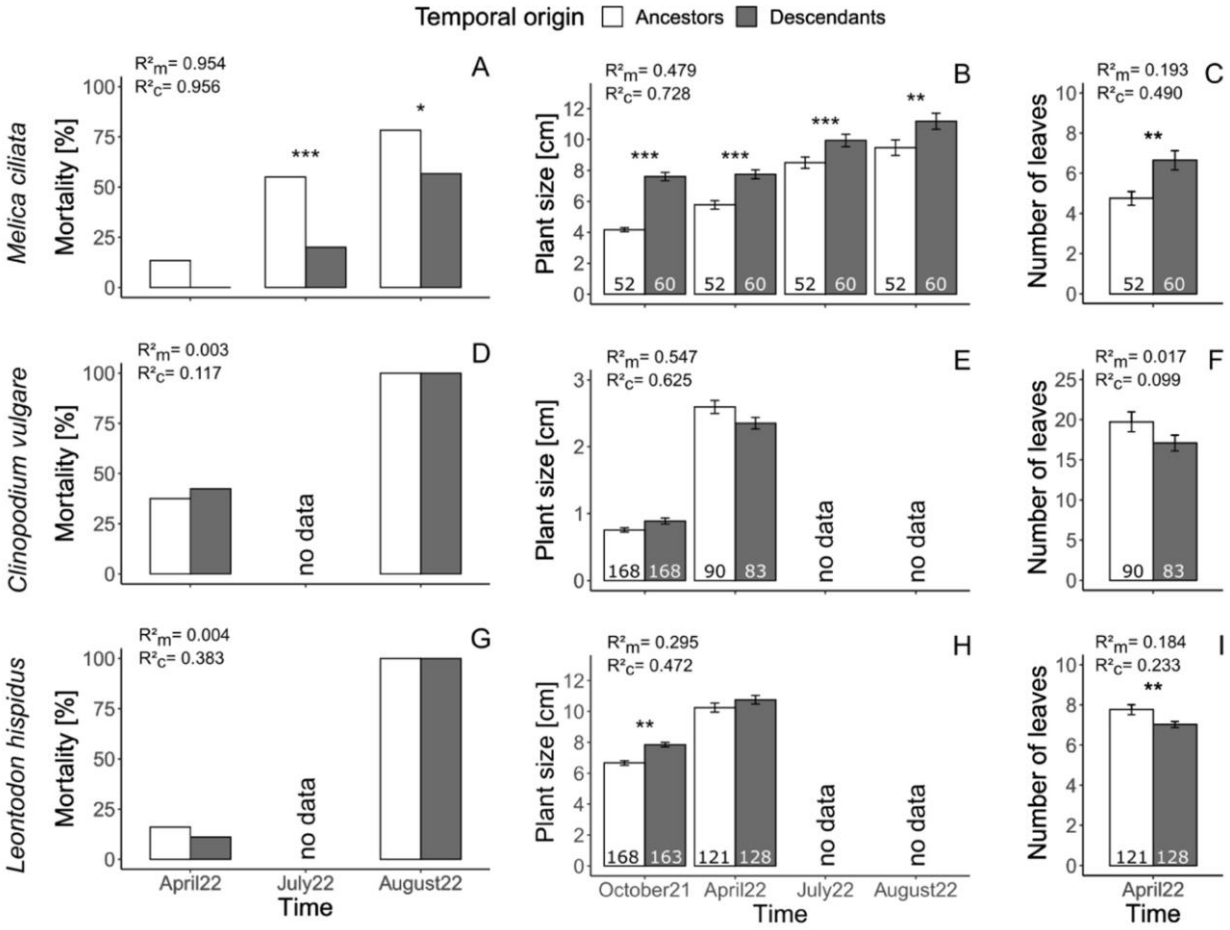

Fig. 3. Mortality rates, plant size and number of leaves of *Melica ciliata* (A, B, C), *Clinopodium vulgare* (D, E, F) and *Leontodon hispidus* (G, H, I) in ancestors (white bars) and descendants (grey bars) measured at different time points. Shown are means and standard errors. Significant differences 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. Marginal R^2 (R^2_m) and conditional R^2 (R^2_c) are provided on the top left of each graph.

Table 1
Resultsof the statistical modelstesting the effectsof temporal origin (ancestors,descendants),time of measurement(if measuredmore than once)and their interaction on mortality (GLMM), plant size(LMM) and number of leaves(LMM) of *Melica ciliata*, *Clinopodiumvulgare*and *Leontodonhispidus*.For plant size, an additional LMM wascreated with temporal origin, time of measurement,mortality and their two- and three-way interactions. Shown are F-valuesand p-valuesfor LMMsand Chi^2 and p-values for GLMMs. Significant p-values(<0.05) are shown in bold.

Responsevariable	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	0.79	0.375
	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 x Time	6.63	**<0.001**	7.83	**0.006**	4.47	**0.035**
Leaf number	Origin	0.38	0.539	2.19	0.152	11.89	**0.002**
	Initial size	16.11	**<0.001**	0.04	0.845	46.26	**<0.001**
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				

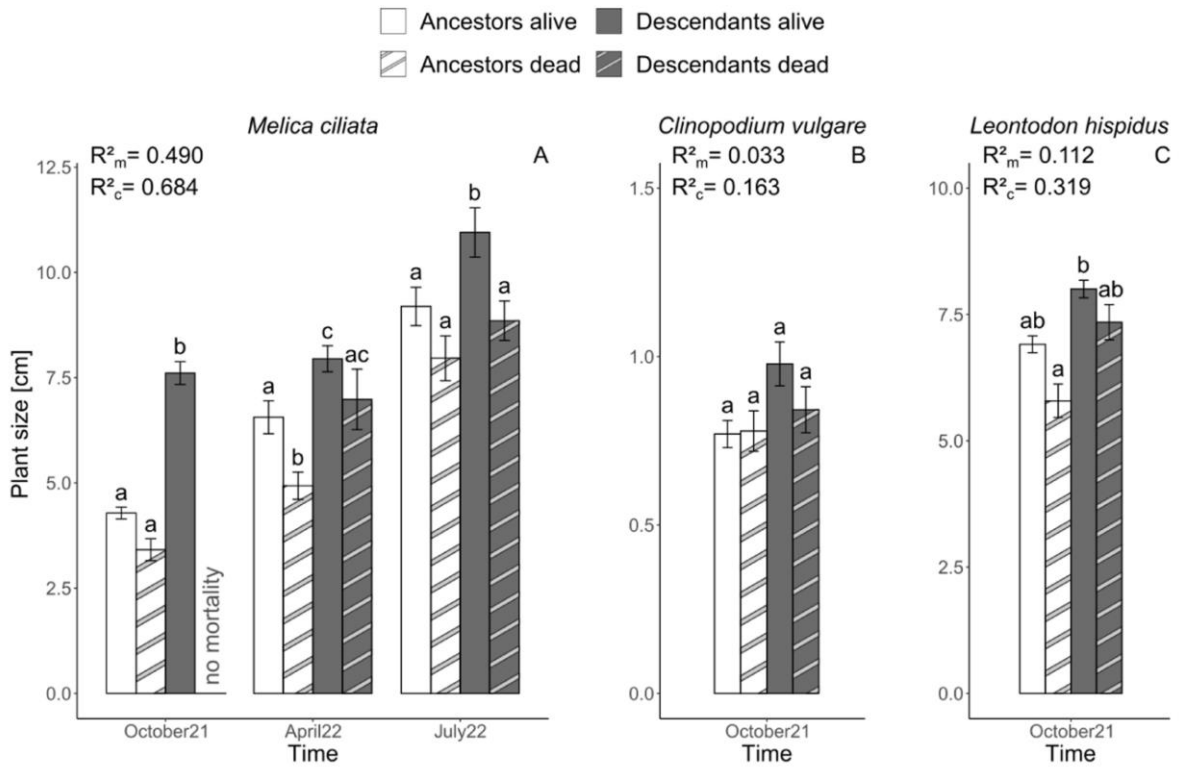

Fig. 4. Plant size of *Melica ciliata* (A), *Clinopodium vulgare*(B) and *Leontodonhispidus*(C) grouped by their mortality status at the next measurement(dead indicated with stripes and alive indicated without stripes) of ancestors(white bars) and descendants(grey bars) at three different time points. Shown are means and standard errors. Significant differencesin plant size between groupsare shown with different letters within eachtime 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 analysed. Marginal R^2 (R^2_m) and conditional R^2 (R^2_c) are provided on the top left of each graph.

2022, descendants carried 6.65 ±0.47 leaves and ancestors had 4.75 ±0.34 leaves, but the differences were mainly driven by initial size ($p < 0.001$, Fig. 3C, Table 1) and not by temporal origin ($p = 0.539$, Table 1).

In *C. vulgare*,mortality did not differ between temporal origins in April 2022 ($p = 0.339$, Table 1) and reached100% 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 at the previous measurements 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: $p = 0.5955$).

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	4.720	**0.030**	1.103	0.294
	Time			1.935	0.380
	Origin×Time			8.666	**0.013**

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, but the extent and direction of changes were species-specific. We found greater plant size 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

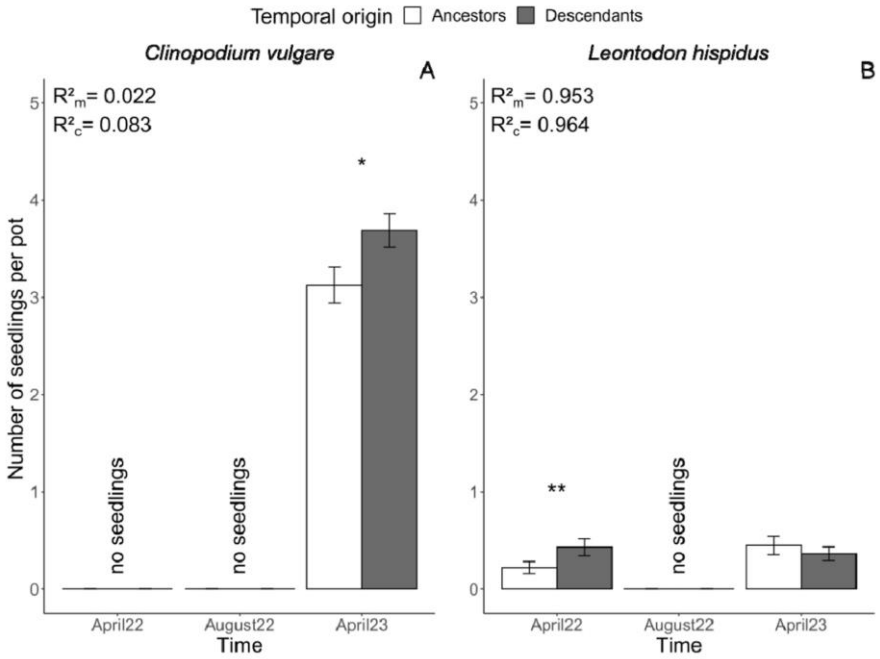

Fig. 5. Number of seedlings germinated per pot from *Clinopodium vulgare* (A) and *Leontodon hispidus* (B) in ancestors (white bars) and descendants (grey bars) measured at different time points. Shown are means and standard errors. Significant differences between ancestors and descendants are indicated with asterisks ($p = 0.05 - 0.01$ *; $p = 0.01 - 0.001$ **). Marginal R^2 (R^2_m) and conditional R^2 (R^2_c) are provided on the top left of each graph.

water availability during this year. Seeds of *L. hispidus* managed to germinate in April 2022 and we recorded more descendant seedlings (4.3% of seedssown) compared to ancestors (2.2% of seedssown). Thus, descendant seedsseem 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. 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 seedssown) compared to ancestors (31.3% of seedssown). 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 both ancestor and descendant seeds had substantial germination but descendant seedlings had lower mortality compared to ancestors before we documented their germination. Even though we cannot pinpoint the underlying cause with certainty, our findings show that the descendant seeds were favoured in the contemporary environmental conditions which is likely due to evolutionary adaptations of the population.

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. S3 H). 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 5–7 °C above the maximum temperature of the previous 30 years, while maximum temperatures in April 2022 were 1–3 °C lower than the 30-year-average (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. Furthermore, the highest mortality increases for ancestors occurred between April and July 2022 (41.7% points), and for descendants between July and August 2022 (36.7% points), indicating that ancestors may have 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. However, differences between generations could also be due to maternal or storage effects, as we were unable to produce a refresher generation for this species (Franks et al., 2018).

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), but might also be due to the bias introduced by the missing refresher generation. 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 maladaptation of the population due to inbreeding or genetic drift (Crespi, 2000) or as a response to dry conditions as it reduces water loss by transpiration and reduces resource needs. The latter 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 did not detect differences in mortality between temporal origins, which may be explained by the exceptional weather conditions during the growing season, because 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).

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 – 295 m) and the soil conditions are similar (Table S4), we can discuss the results with respect to evolutionary adaptation. We also investigated rather isolated populations, allowing us to attribute trait differentiation to evolutionary change by natural selection on standing variation rather than by immigration (Pluess, 2013).

4.2. Limitations and future directions

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 pressure sacted. 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.

Our approach revealed evidence for rapid adaptations in our study species. However, our study has some limitations that could affect the robustness of our conclusions. First, the inclusion of refresher generations differed among species, with no refresher generation for *M. ciliata*, one refresher generation for *C. vulgare*, and two refresher generations for *L. hispidus*, therefore potentially varying in their degree of non-genetic influences on the measured phenotypes. Especially the validity of results for *M. ciliata* is potentially compromised, since the seed quality of the ancestral seeds might be negatively affected by the storage time or influenced by maternal effects (Franks et al., 2018). Another potential confounding factor for the interpretation of our results 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* while the influence on *C. vulgare* might be limited as well, given that newly sampled seeds had a similar germination rate (40%).

Ideally, we would have transplanted plants within the original population, but in order to avoid confusion of our study plants with wild individuals of the same species, we selected transplantation plots in the vicinity of the original population where no natural individuals were present. Although, the plots were in the same habitat and soil characteristics were similar, the original population might not inhabit these nearby plots due to slightly unfavorable microclimates or competition with resident plants. Moreover, we had to mow the transplantation sites which may have changed the microclimate (e.g., shading) compared to the original sites. For those reasons, we recommend for future studies to transplant individuals directly into the original population, avoid

mowing if possible,and take great care to mark the study plantsto avoid confusion. Likewise, our experimental seed-sowing setup, although necessaryto ensure that the sowed seedswould not be washed away by rain or predated, did not fully represent natural conditions. Especially the placement of the protective net could have further reduced the amount of precipitation received by the seedsduring an already dry period and the low volume of the pots could facilitate drying out, exacerbating the already dry conditions.

Despite the limitations of our study, 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 speciesneed 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 responsesdue 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 multiple descendant generations, ideally a series of generations, to investigate the natural fluctuation in phenotypic expression across generations. This is important becauseit is often assumed that evolutionary changesbuild up gradually over time whereas these could also be the result of extreme selection events in single years (Gould and Eldredge, 1977). Finally, we suggestto include multiple populations of the samespecies,if these are available, to allow more general statements about the evolutionary potential of the species.

5. Conclusion

By combining a resurrection experiment with in situ transplantations, we found evidence for species-specificevolutionary adaptationsafter 23 –26 yearsof climate change. Resultson *M. ciliata* suggest evolution of local adaptation to drought conditions, and evidence of evolutionary adaptations to drought in seed traits were found for *C. vulgare*and *L. hispidus*.Thesefindings further support the notion that drought has been a key selective force for evolution in recent years,and that current adaptations to prolonged drought periods will play an important role for persistenceof plant populations in the future (Franks et al., 2007; Nevo et al., 2012; Rauschkolb et al., 2023; Sekor and Franks, 2018; Thomann et al., 2015; Vigouroux et al., 2011). Evolutionary adaptations are likely the result of drought-tolerance functional strategies (Basuet al., 2016), but also the evolution of seedtraitssuch as dormancy and faster germination can be beneficial (Volaire and Norton, 2006). Our study approach 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.

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CRediT authorship contribution statement

PK: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. **AE:** Writing – review & editing, Conceptualization, Methodology, Validation. **MMS :** Formal analysis, Investigation, Supervision, Validation, Writing – review & editing, Software, Conceptualization, Methodology. **SG:** Investigation, Methodology, Resources,Supervision, Writing – review & editing, Conceptualization, Project administration, Validation. **RR:** Methodology, Resources,Writing – review & editing, Conceptualization, Validation. **JFS:** Formal analysis, Investigation, Resources,Supervision, Validation, Writing – review & editing, Methodology, Conceptualization, Visualization.

Author statement

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

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw data that supports the findings of this study are available at Dryad (https://doi.org/10.5061/dryad.sj3tx96bf).

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Appendix A. Supporting information

Supplementary data associatedwith this article can be found in the online version at doi:10.1016/j.ppees.2023.125773.

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Supplement material – Chapter I

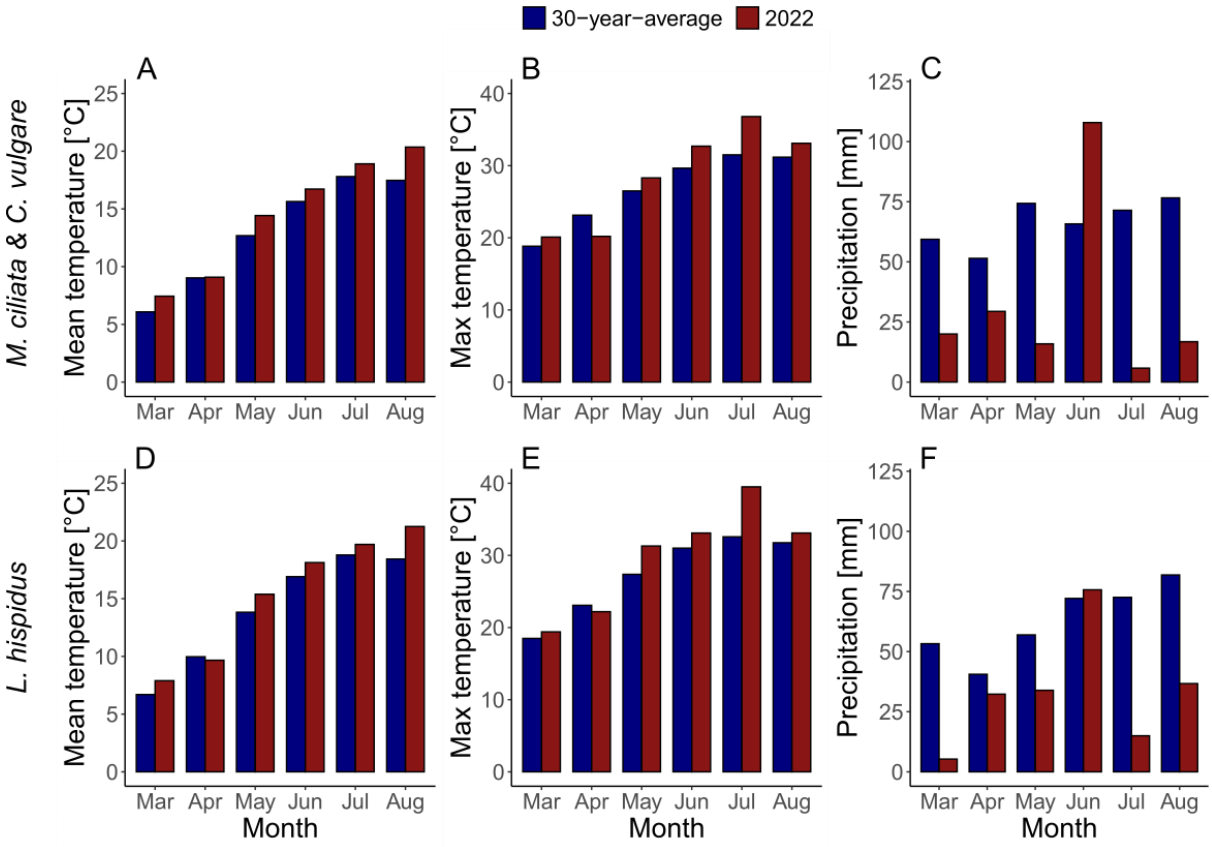

Figure S1. Climate data from a weather station near *Melica ciliata* and *Clinopodium vulgare* (A, B & C) and a weather station near *Leontodon hispidus* (D, E & F) showing mean temperature [°C], max temperature [°C] and precipitation [mm] during the months May – August. The average of 1989 – 2019 (30-year average) is shown in blue bars and the year 2022 is shown in red bars. The weather station of Dourbes is located 3 km from the *M. ciliata* and 14 km from the *C. vulgare* population. The weather station of Maastricht is located 14 km from the *L. hispidus* population.

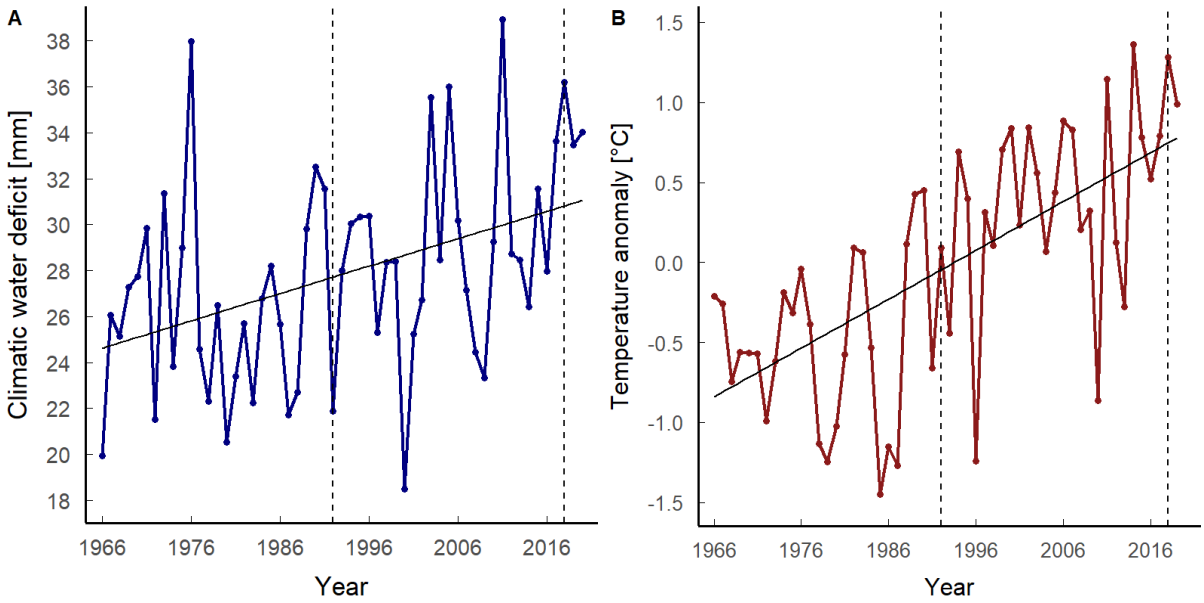

Figure S2. Temperature anomaly (A) and climatic water deficit (B) over the last four decades of *Melica ciliata* and *Clinopodium vulgare* populations. The dotted lines refer to the seed collection years (ancestors 1992 vs. descendants 2018).

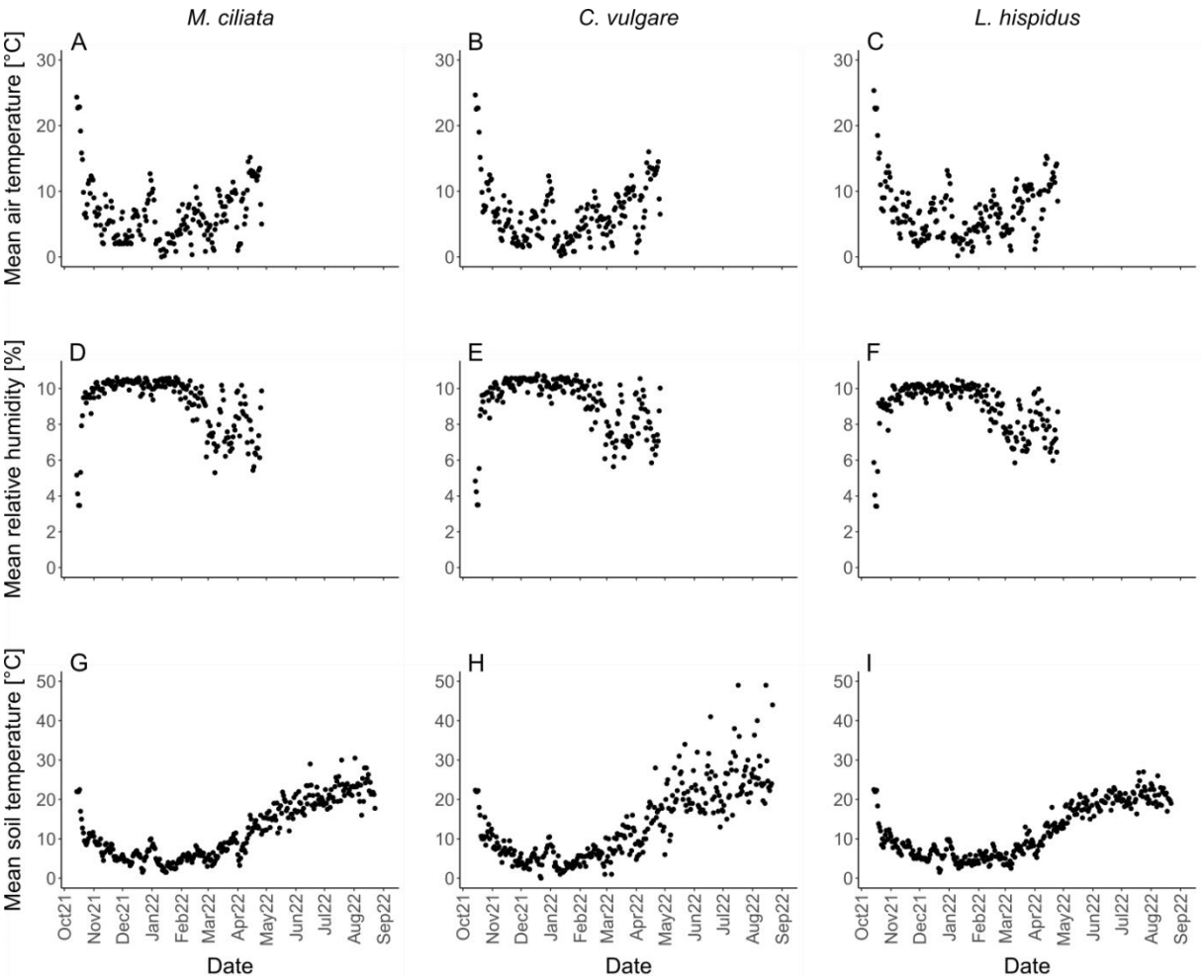

Figure S3. Daily mean values of air temperature [°C] relative humidity [%] and soil temperatures [°C] for *Melica ciliata* (A, D, G), *Clinopodium vulgare* (B, E, H) and *Leontodon hispidus* (C, F, I) during the experimental period from October 2021 to September 2022. Air temperature and relative humidity was measured by a shaded data logger (iButton DS1923, Maxim Integrated) in the center of each plot. Mean soil temperature was measured by two data loggers (iButton DS1921G-F5, Maxim Integrated) buried 5 cm deep into the soil in two opposing corners of each experimental plot. All data loggers recorded one value every 4 hours. Data for relative humidity and air temperature only extends until April 2022, because the loggers malfunctioned afterwards.

Table S1. Coordinates, collection years and number of seed families of the study species.

Species	Coordinates	Collection years	Number of seed families in 2018/2020	Estimated population size in 2018
Clinopodium vulgare	50°03'55"N 4°26'40"E	1992/2018	47	500
Melica ciliata	50° 4' 34" N 4°32'35"E	1992/2020	21	25
Leontodon hispidus	50°47'35"N 5°40'25"E	1995/2018	20	100

Table S2. Results of the statistical models for *Melica ciliata* testing the effects of the dataset (subset vs. complete) on mortality, plant size and number of leaves. The model for the number of leaves (LMM) contained no other explanatory variables, whereas the model for plant size (LMM) and for mortality (GLMM) included the time of measurement and the dataset × time interaction. Shown are F-values and p-values for the LM and LMM and Chi^2- and p-values for the GLMM. Significant p-values (< 0.05) are shown in bold.

Response variable	Explanatory variable	*M. ciliata*	
		Chi^2	p
Survival	Dataset	0.01	0.935
	Time	21.55	**< 0.001**
	Dataset × Time	54.98	**< 0.001**
		F	p
Plant size	Dataset	0.23	0.630
	Time	85.46	**< 0.001**
	Dataset × Time	0.03	0.994
Ramets	Dataset	0.01	0.915
	Time	32.51	**< 0.001**
	Dataset × Time	0.07	0.934

Table S3. Results of the statistical models testing the effects of time of measurement (only *M. ciliata* due to multiple measurements), plant size and their interaction on mortality (GLMM) of *Melica ciliata, Clinopodium vulgare* and *Leontodon hispidus*. Shown are F-values and p-values. 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
Mortality	Time	31.256	**< 0.001**				
	Plant size	22.334	**< 0.001**	1.594	0.207	9.481	**0.002**
	Time × Plant size	3.515	0.172				

Table S4: Chemical composition of soil samples taken at the collection sites and the transplantation sites of our study species *Melica ciliata, Clinopodium vulgare* and *Leontodon hispidus*. We took four samples of 25 cm² soil each at 10 cm depth at random positions in each site. The four samples from each location were mixed together and analysed 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.

Species	Site	pH	Salinity [µS/cm]	N [%]	C [%]	TIC [%]	C org [%]	C/N	S [%]	Ca [mg/kg]	K [mg/kg]	Mg [mg/kg]	P [mg/kg]
M. ciliata	Collection	7.45	308	1.28	15.47	1.02	14.45	11.26	0.04	53876	11351	3990	1294
	Transplantation	7.13	251	0.95	12.36	0.16	12.2	12.81	0.04	22518	8010	3742	935
C. vulgare	Collection	6.56	114	0.53	6.45	0.01	6.44	12.17	0.02	4124	29625	9811	569
	Transplantation	6.62	88	0.43	4.6	0.04	4.56	10.67	0.02	4509	28270	9213	710
L. hispidus	Collection	6.46	88	0.49	6.9	0	6.9	14.14	0.01	3966	8608	1090	569
	Transplantation	6.52	127	0.76	11.01	0.01	11	14.51	0.03	5950	7252	1048	710

Chapter II

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

Status: submitted

Contributing Authors: P. Karitter, E. Corvers, M. Karrenbauer, M. March-Salas, B. Stojanova, A. Ensslin, R. Rauschkolb, S. Godefroid, J.F. Scheepens

What are the contributions of the doctoral candidate and the co-authors?

1. Concept and design
The experimental concept was developed by the doctoral candidate (40%), AE (5%), RR (5%), SG (15%) and JFS (35%). The experimental design was planned by the doctoral candidate (60%), MMS (10%), and JFS (30%).
2. Conducting tests and experiments
Measurements of the experiment were performed by the doctoral candidate (20%), EC (40%), and MK (40%).
3. Compilation of data sets and figures
Data were compiled and illustrations prepared by the doctoral candidate (80%) and EC (10%) and MK (10%).
4. Analyses and interpretation of data
Data were statistically analysed by the doctoral candidate (55%), EC (5%), MMS (20%), and JFS (20%). Data interpretation was performed by the doctoral candidate (45%), MMS (20%), AE (5%), RR (5%), SG (5%), and JFS (20%).
5. Drafting of manuscript
The first draft of the manuscript and all subsequent revisions were made by the doctoral candidate (50%). Additional contribution and comments were made by MMS (10%), BS (10%), AE (5%), RR (5%), SG (5%), and JFS (15%).

I hereby certify that the information above is correct.

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 (p.karitter@gmail.com)

Declaration of Authorship[1]

[1]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.

Abstract

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 and force them to optimize resource use against competitors. To date, the evolution of competitive ability in the context of changes in nutrient availability remains understudied. In this study, we investigated whether the common calcareous grassland herb *Leontodon hispidus* recently evolved its competitive ability and response to nutrient availability. We compared ancestors sampled in 1995 and descendants sampled in 2018 and applied a competition treatment in combination with weekly nutrient treatments (no fertilizer, nitrogen, phosphorus, and both). 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. Our study demonstrates rapid contemporary evolution of competitive ability, 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.

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 (Newman 1995; Smith et al. 1999; Galloway et al. 2008). 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 (Mosquin 1971; Bonser and Ladd 2011; Gao et al. 2022).

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 (Newman 1995; Smith et al. 1999; Galloway et al. 2008). 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 (Djodjic et al. 2004; BDB 2005; 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 and Dybzinski 2013), changes in the availability of these resources may affect the evolution of plant responses since less competitive species are likely to experience higher mortality (Grime 1973). An increase in soil nutrient resources in a nutrient-limited habitat 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 and 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 and Blatt 2009). Consequently, changes in nutrient supply and resulting impacts on competition can impose strong selection pressure on plants to evolutionarily increase either their stress-tolerance or competitive ability through adjusting growth-related traits under the novel environmental conditions (Falster and Westoby 2003; Craine and Dybzinski 2013). 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 (Sultan et al. 2013; Frachon et al. 2017; 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. In calcareous grasslands, biodiversity is threatened by the increasing dominance of the grass *Brachypodium pinnatum* (Bobbink and Willems 1987; Bą ba 2003; Canals et al. 2017) and evolution of competitive ability could be essential for the persistence of some plant populations in this habitat. 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 and 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: Seminatural 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). The germination success of ancestral seeds was very high with 93 % and thus, the likelihood of artificial selection during storage (i.e., invisible fraction) is low (Weis 2018). 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 nutrientpoor 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, 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 centre 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 counted (Online Resource 1) and 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 and 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 (Online Resource 2). 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). Posthoc 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 population 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 and 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 and 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 (Klein et al. 2019; European Environment Agency 2021). The total P content on the other hand was 530 mg/kg in our studied site (Online Resource 2) 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; Wilson and 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 used nine seed families in our study which might not fully represent the genetic diversity of the population. Moreover, we had little data available on the local changes of relevant environmental factors that the ancestors and descendants experienced. Finally, we only studied a single population of *L. hispidus*, making it difficult to generalize the results to the species level.

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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Declarations

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Conflict of interest The authors declare that they have no conflict of interests.

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

Author contributions 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.

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Tables

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

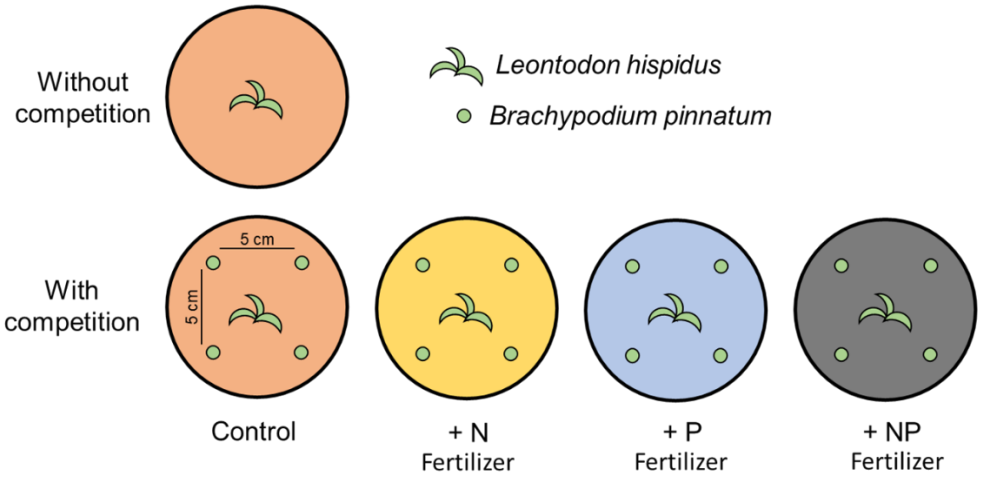


Figure 1. Experimental design of the study. Ancestors and descendants of *Leontodon hispidus* were cultivated in pots and divided into 5 treatment groups. One group was cultivated without competition and no additional nutrient supply. The other four groups were all grown with competition in combination 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.

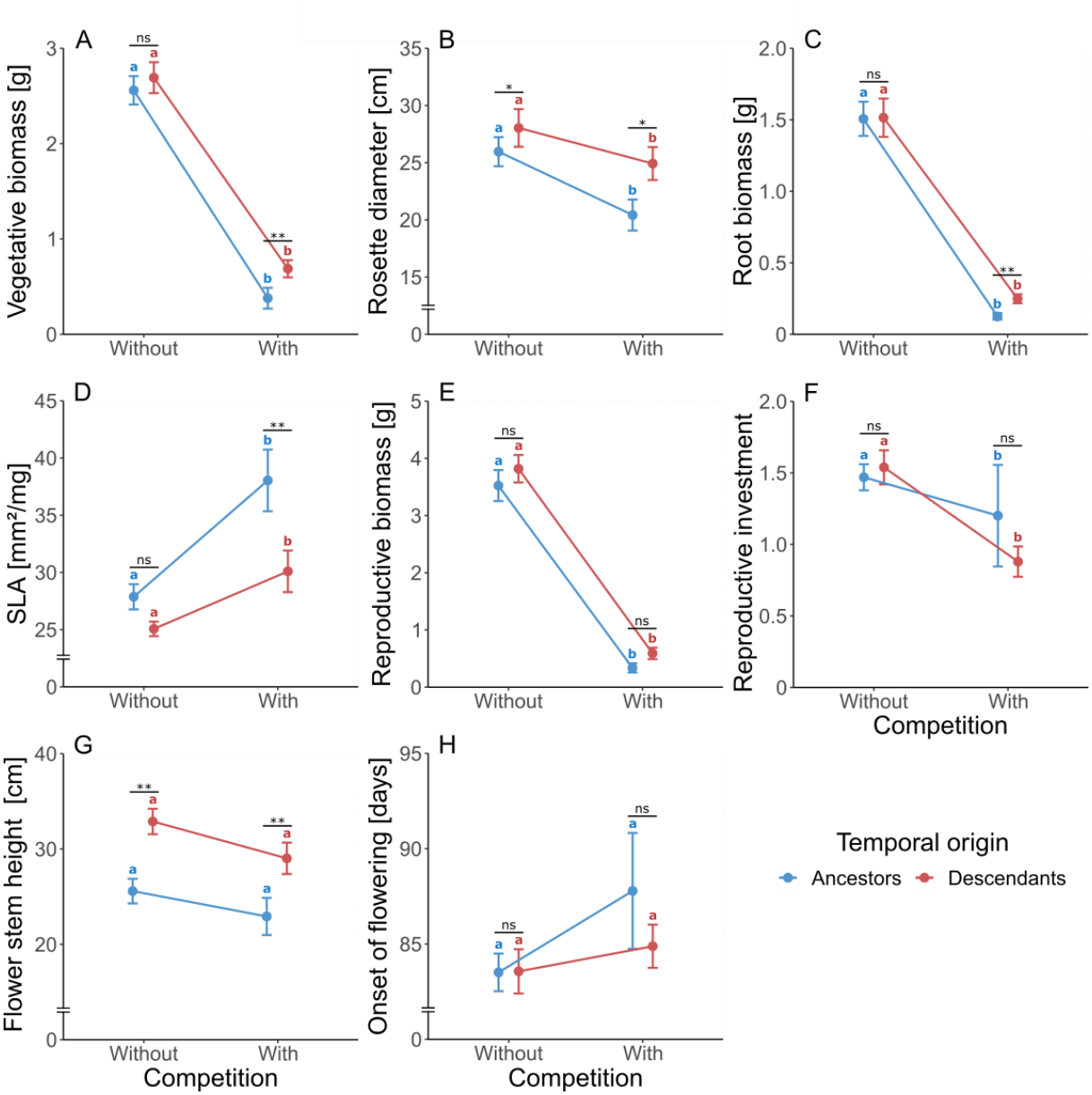


Figure 2. Vegetative biomass (A), rosette diameter (B), root biomass (C), specific leaf area (D), reproductive biomass (E), reproductive investment (F), flower stem height (G) and onset of flowering (H) of ancestors (blue) and descendants (red) of *Leontodon hispidus* grown either without competition 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).

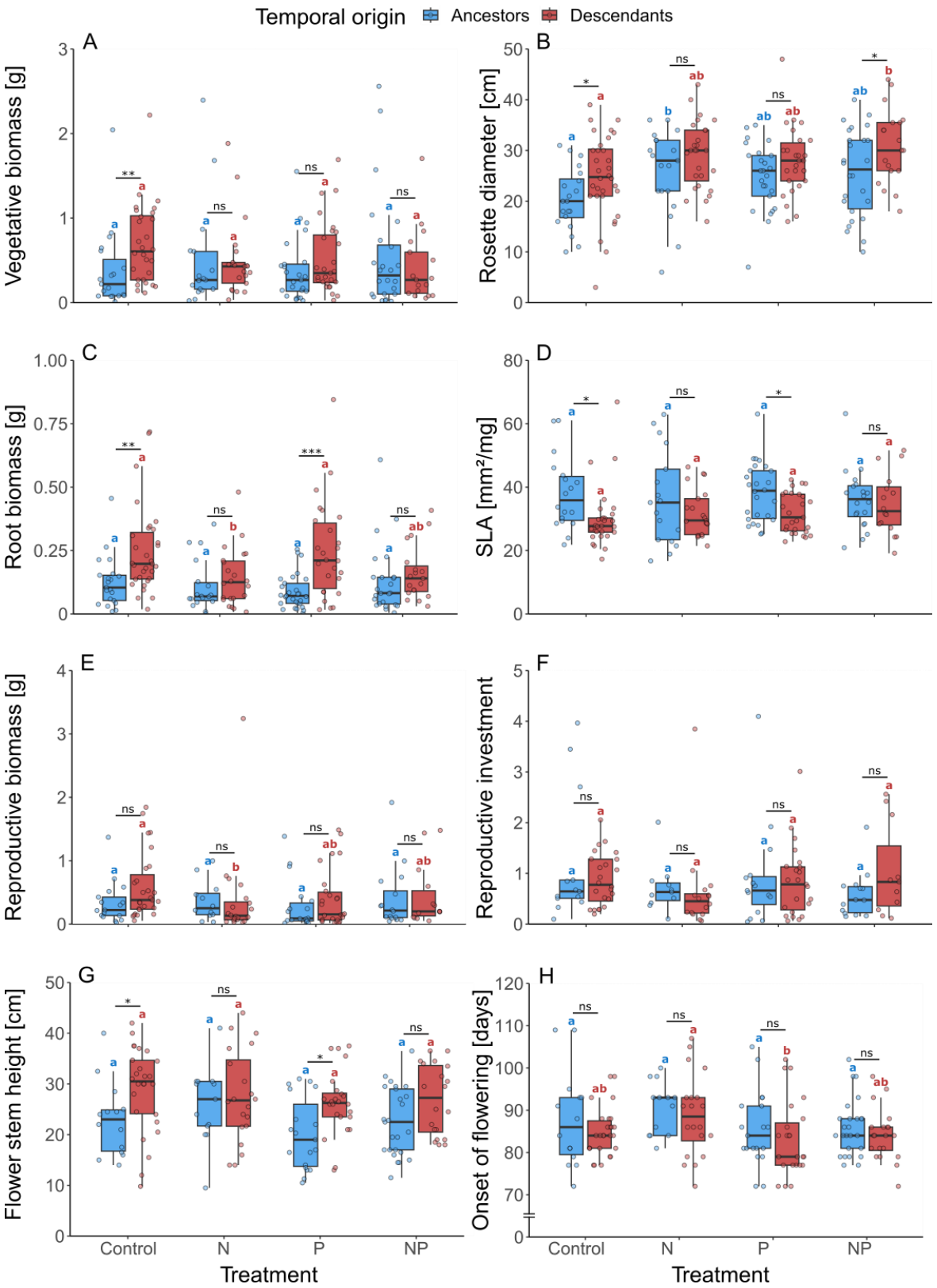
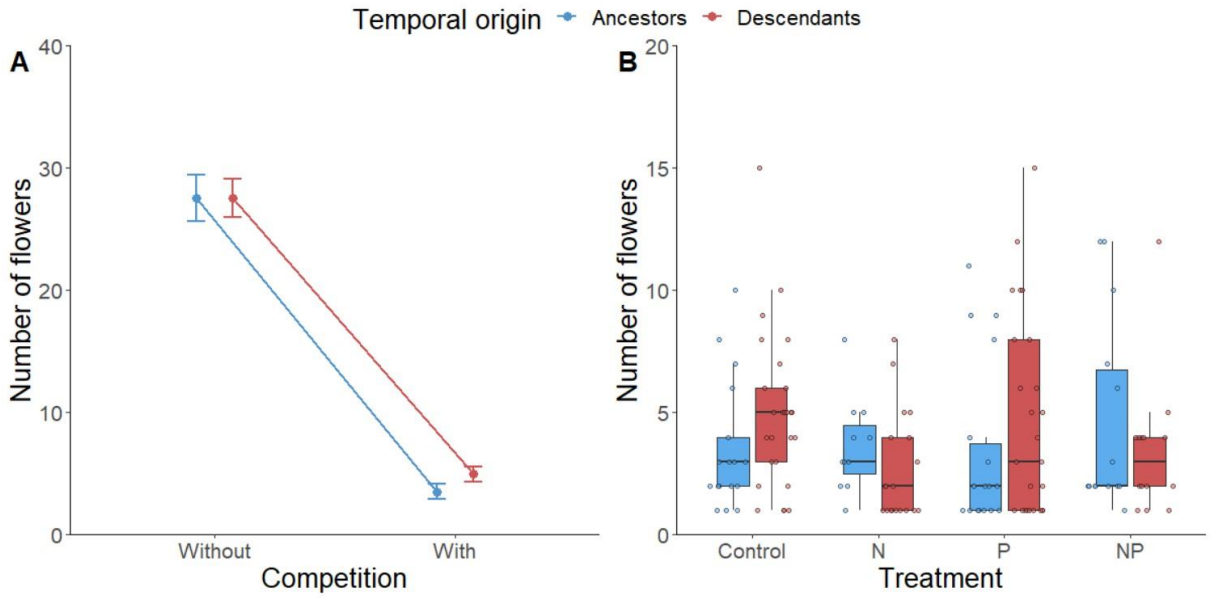


Figure 3. Vegetative biomass (A), rosette diameter (B), root biomass (C), specific leaf area (D), reproductive biomass (E), reproductive investment (F), flower stem height (G) and onset of flowering (H) of ancestors (blue) and descendants (red) of *Leontodon hispidus* grown under different nutrient treatments (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).

Supplement material (Online Resources) – Chapter II



Online Resource 1. 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).

Online Resource 2. 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 analysed 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

Chapter III

Declaration of author contributions to the manuscript: Garden, greenhouse or climate chamber? Experimental conditions influence whether genetic differences are phenotypically expressed

Status: submitted

Contributing Authors: P. Karitter, M. March-Salas, A. Ensslin, R. Rauschkolb, S. Godefroid, H. Poorter, J.F. Scheepens

What are the contributions of the doctoral candidate and the co-authors?

1. Concept and design
The experimental concept was developed by the doctoral candidate (65%), and JFS (35%). The experimental design was planned by the doctoral candidate (55%), MMS (20%), and JFS (25%).
2. Conducting tests and experiments
Measurements of the experiment were performed by the doctoral candidate (100%).
3. Compilation of data sets and figures
Data were compiled and illustrations prepared by the doctoral candidate (100%).
4. Analyses and interpretation of data
Data were statistically analysed by the doctoral candidate (55%), MMS (20%), and JFS (20%). Data interpretation was performed by the doctoral candidate (50%), MMS (20%), HP (10%) and JFS (20%).
5. Drafting of manuscript
The first draft of the manuscript and all subsequent revisions were made by the doctoral candidate (45%). Additional contribution and comments were made by MMS (10%), AE (5%), RR (5%), SG (5%), HP (15%) and JFS (15%).

I hereby certify that the information above is correct.

Garden, greenhouse or climate chamber? Experimental conditions influence whether genetic differences are phenotypically expressed

Pascal Karitter[1*], Martí March-Salas[1], Andreas Ensslin[2], Robert Rauschkolb[3,4], Sandrine Godefroid[5], Hendrik Poorter[6,7], Johannes F. Scheepens[1]

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

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

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

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

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

[6]Horticulture and Product Physiology, Wageningen University, Droevendaalsesteeg 1, 6708PB Wageningen, the Netherlands

[7]Department of Biological Natural Sciences, Macquarie University, North Ryde, NSW 2109, Australia

[*] Corresponding author: Pascal Karitter (p.karitter@gmail.com)

Conflict of interest

The authors have no conflict of interest to declare.

Author contribution

PK, MMS and JFS designed the study. SG and RR provided the seed material. PK conducted the experiment. PK, MMS and JFS analysed the data. PK wrote the first draft and all the authors contributed to further versions of the manuscript.

Abstract

1. Common-environment experiments are important to study genetically-based phenotypic variation within and among plant populations. Such experiments can be performed in an experimental garden, greenhouse or climate chamber. However, phenotypic expression may be strongly affected by the environmental conditions and influenced by parental and storage effects. Hence, it is unclear if results from common-environment experiments are reproducible across multiple experimental setups.
2. In this study, we assessed the effects of three different growth facilities – outdoor garden, greenhouse, and climate chamber –, on phenotypic expression. We compared ancestral and descendant genotypes of the same population of *Leontodon hispidus*. We also evaluated differences in phenotypic expression between plants grown after one (F1) vs. two (F2) intermediate generations.
3. We observed strong differences among plants growing in different growth facilities. Furthermore, we found that descendants had larger rosettes than ancestors only in the greenhouse and they flowered later than ancestors exclusively in the climate chamber. We did not find significant differences between intermediate generations within the growth facilities.
4. Overall, our study demonstrates that environmental variation among growth facilities can dictate the presence and magnitude of phenotypic differences. This implies that absence of evidence for phenotypic differences is not evidence of absence. Experimental systems should be carefully designed to provide meaningful conditions related to the research question. Finally, growing a second intermediate generation did not impact the genetic differences of ancestors and descendants within the facilities, supporting that only one intermediate generation may be sufficient to reduce detectable parental and storage effects.

Introduction

Common-environment experiments have been a powerful approach for evolutionary biologists ever since the Swedish botanist Göte Turesson established this approach one century ago. The ability of a given genotype to express different phenotypes depending on the environment (i.e., phenotypic plasticity; Sultan, 2000) complicates studies on the genetic basis of phenotypic differences, since field observations cannot be used to unravel genetic from plastic effects on the phenotype. However, by growing plants of the same species from different habitats in the same developmental environment, Turesson (1922) was able to attribute phenotypic variation among plant populations to genetic differences and identify natural selection as the main driver. This concept was further developed to study local adaptation by transplanting individuals originating from different populations reciprocally among the habitats from which they were sampled (Clausen et al., 1940; Kawecki and Ebert, 2004). Common-environment experiments can even be used to study rapid evolution of plant populations to recent environmental changes by experimentally applying different selection pressures over multiple generations (Hill and Caballero, 1992) or by using the resurrection approach, in which seeds collected before a potential selection pressure are revived and compared to plants from recently collected of the same population (Franks et al., 2018). Comparisons of these phenotypes in a common environment can then uncover evolutionary changes that occurred between samplings (Franks et al., 2007).

It is important that the results of experimental approaches are reproducible and robust to ensure their validation and generalization, but this can be challenging (Drummond, 2009). Plant responses can be affected by the choice of the growth facility (i.e., outdoor garden, greenhouse or climate chamber; Poorter et al., 2016) or non-genetic biases such as parental effects (Latzel et al., 2023). For instance, Massonnet and colleagues (2010) studied leaf growth variables and other traits of three *Arabidopsis thaliana* genotypes in 10 different laboratories and found that modest variations in growing conditions such as temperature, light quality and the handling of the plants can induce significant differences in molecular profiles and phenotypes. Consequently, relatively small differences in the growth facility can lead to significant differences in plant responses and results can be strongly affected by the choice of the common environmental conditions. Poorter and colleagues (2012) described

experimental outdoor gardens as being relatively close to natural conditions in the field. They characterized outdoor gardens as commonly having low spatial heterogeneity but high temporal variations in temperature, light and water supply, high chance of plant damage (hail, herbivory, late frost) and episodes of extreme conditions (e.g., high irradiance causing high temperatures, and low precipitation). According to Poorter and colleagues (2012), greenhouses normally provide more buffered conditions with heating systems to protect against frost, adjustable shading screens to counteract high irradiance, and with a more controlled water supply. However, air temperatures can peak depending on the ventilation system, and the greenhouse can substantially shade the plants due to structural elements (Cabrera-Bosquet et al., 2016). In climate chambers, researchers can control the experimental conditions as reliable as possible, which would be optimal for reproducibility, but the conditions can be quite artificial and deviate strongly from field conditions with more heterogeneous light and humidity distributions. Especially light can have strong vertical gradients and heterogeneous horizontal gradients (Poorter et al., 2012). Furthermore, climate chambers provide limited space which can greatly affect the possible sample size for larger species and thus, have a trade-off between control over the environment and statistical power. These differences among growth facilities may be important, as certain environmental conditions may not elicit phenotypic variation, even though genetic variation is present among genotypes. Accordingly, Stanton and colleagues (2000) found that, compared to near-optimal growing conditions, more stressful conditions, which better mimic natural conditions, tend to increase phenotypic variation among genotypes of wild mustard (*Sinapis arvensis*). Consequently, is unclear if the results from common-environment experiments are consistent throughout different experimental environments and can be accurately reproduced. If not, the consequence would be that we may miss evidence of relevant genetic variation or, in contrast, that we may detect genetic variation that has currently no adaptive relevance in the field.

Studies on genetic differentiation can also be confounded by non-genetic or random variability induced by parental effects or storage. Parental effects occur, when the parental phenotype affects the phenotype of their offspring irrespective of the genes that are inherited (Auge et al., 2017; Badyaev and Uller, 2009). Seed provisioning is one major parental effect that can have a big impact on the offspring, because the resource availability and general environmental conditions (e.g., light

quantity, quality and duration) of the mother plant can determine the amount of resources for the seeds and consequently affect seedling establishment and early life history traits (Herman and Sultan, 2011). Other parental effects include hormone-driven effects on physiology of the seedling or epigenetic processes through passing on distinct DNA methylations or chromatin changes (Blödner et al., 2007; Elwell et al., 2011; Herman and Sultan, 2011; Jablonka and Raz, 2009; Richards et al., 2017). Even though parental effects can have ecological and evolutionary significance (Latzel et al., 2023), their influence can be a source of bias in studies on genetic differentiation when parental environmental conditions differ among genotypes (Bischoff and Müller-Schärer, 2010). Furthermore, long storage periods can affect seed viability and even post-emergence traits (Franks et al., 2018b). In experiments where plants from the same population but largely different generation are compared, so-called 'resurrection experiments', storage duration and conditions may well have been different. Consequently, phenotypes of seedlings may express varying plastic responses (Weis, 2018). These biases can be minimized by acclimating the experimental plants under common environmental conditions for one or more generations before the start of the experiment (Kawecki and Ebert, 2004). Parental effects caused by the environment usually disappear after one generation in a new environment (Agrawal, 2002; Gianoli, 2002), but have also been shown to persist over multiple generations (Wulff et al., 1999). Latzel (2015) recommends at least two intermediate generations before the start of an experiment, as it increases the chance of evening out epigenetic modifications. Still, the method of growing intermediate generations is not always implemented in common-environment studies as it is time- and labor-intensive, especially if the study plants do not flower in the first year (Bischoff and Müller-Schärer, 2010; Rauschkolb et al., 2023).

In this study, we investigated the consistency of phenotypic differences of a resurrection common-environment experiment among different growth facilities. Furthermore, we tested for differences between results from experimental plants grown after one vs. two intermediate (i.e., refresher) generations. Absence of any differences would suggest that one intermediate generation is sufficient to reduce detectable parental, epigenetic or storage effects. We worked with the perennial *herb Leontodon hispidus* and used seeds of ancestors sampled in 1995 and of descendants sampled in 2018 collected from the same population in a calcareous grassland in Belgium. We

grew two intermediate generations (F1 and F2) from both ancestors and descendants under common conditions and cultivated them in three different growth facilities: in an outdoor garden, in a greenhouse and in a climate chamber. We measured eight traits regarding growth, leaf anatomy and flowering phenology to cover a wide spectrum of functional traits. We hypothesized that (1) genetically-based phenotypic differences across traits tend to occur inconsistently across substantially different growth facilities. (2) We expected that phenotypic differences in evolved traits between ancestors and descendants would be strongest in less-controlled conditions such as the outdoor garden and weakest in the more optimal and constant conditions in the climate chamber. Finally, we hypothesized that (3) one intermediate generation is not enough to sufficiently reduce non-genetic differences between ancestors and descendants, which may be attributed to the storage or parental effects of ancestors.

Material and Methods

Study species

Leontodon hispidus (Asteraceae) is a perennial herbaceous herb and typically flowers from June to October (Kühn and Klotz, 2002). It is self-incompatible and pollinated by insects (Kühn and Klotz, 2002). It is widespread throughout Europe and commonly found in calcareous grasslands. Seed material was collected from two temporal origins, 1995 (ancestors) and 2018 (descendants), from a single population in a dry calcareous grassland in a Belgian nature reserve (50°47’35″N, 5°40’25″E). The distance to the nearest neighboring population is approximately 2 km, likely preventing the majority of gene flow into the population. The staff of the Meise Botanic Garden (Belgium) collected the ancestral seeds for conservation purposes and efforts were made to represent the genetic diversity of the population by collecting from as many individuals as possible dispersed throughout the population. The seed material from an unknown number of mother plants was cleaned, bulked, dried at 15% relative humidity, and stored at -20 °C at the seed repository of the Meise Botanic Garden. In the summer of 2018, we revisited the population and collected seeds from 20 mother plants. These seeds were cleaned, bulked and then stored at 4 °C. Rauschkolb et al. (2022a) analyzed genomic relatedness and allelic richness among individuals within

both temporal origins (ancestors and descendants) and found similar levels of relatedness without obvious kinship structure, which supports the comparability of the sampling procedures and confirms that sufficient seeds were collected.

Experimental design

Both ancestral and descendant seeds were grown for two consecutive intermediate generations (F1 and F2). For the first intermediate generation (F1), we sowed 300 seeds from each temporal origin and selected 15 random individuals from each temporal origin which were randomly pollinated by hand in net cages to prevent unintentional cross-pollination (Rauschkolb et al., 2022b). We used the seeds from the F1 intermediate generation for the F2 generation and grew them under similar conditions, this time using bumblebees (Natupol Seeds, Koppert GmbH, Straelen, Germany) as pollinators. Ultimately, seven maternal lines from the F1 intermediate generation and eight maternal lines from the F2 intermediate generation yielded sufficient seed material for both temporal origins. For each maternal line, we used 12 seedlings grown individually in black 1.5 L pots that were prepared as follows: In July 2022, we placed 12 pots for each maternal line with cultivation soil (Spezial Substrat Typ T1b, Hawita GmbH, Vechta, Germany) in the greenhouse and sowed three seeds into each pot. All pots were watered three times a week to maximum soil capacity.

After the seedlings had developed their first true leaf, we thinned them to a single individual per pot, with the seedling moved to the center of the pot. We measured the initial size as rosette diameter and divided the pots randomly into three groups with four individuals from each maternal line. Each group was grown in a different growth facility for the rest of the experiment: outdoor garden, greenhouse or climate chamber. In total, we used 360 plants for this experiment (3 growth facilities × 2 temporal origins × 2 generations × 7/8 maternal lines × 4 replicates). Pots in the garden were placed on gravel 2 m below a shading cloth to reduce radiation and temperature stress (Schattiergewebe 45%, Nitsch GmbH, Kreuztal, Germany). Plants were randomized every two weeks and watered weekly to soil capacity for the duration of the experiment. In the greenhouse, the plants were placed 1 m below lamps with a combination of two fluorescent tubes (Lumilux HO 80W-865, Berlin, Germany and Gro-Lux FH 80W,

Sylvania, Erlangen, Germany). The lamps were programmed to switch on between 6 am and 20 pm (14 h photoperiod) whenever the natural light intensity went below 360 µmol•m^{-2}•s^{-1} outside. To avoid extreme temperatures, sliding shutters and lamps were programmed activate once light intensity surpassed 1100 from µmol•m^{-2}•s^{-1} outside. The climate chamber (ThermoTec GmbH, Weilburg, Germany) was set to 14h – 10h day – night cycle with 21°C during the day and 18°C during night to simulate the start of the growing season. Air humidity was set to a constant 60% and the plants were placed 1 meter below halogen lamps (Radium HRI-BT 400W/D Pro Daylight, Lampenwerk GmbH, Wipperfürth, Germany).

In each growth facility, we inserted four temperature and soil moisture loggers (TMS-4 logger, Tomst, Prague, Czechia) in the center of black 1.5 L pots with the same cultivation soil and positioned them randomly among the pots with plants. The loggers monitored soil temperature at 5 cm depth, soil surface temperature, air temperature at 5 cm above the soil as well as soil moisture every 15 min. We used this data to calculate mean values of all four loggers from each environment and to derive daily mean values for all parameters (Fig. 1, Appendix S1; see Supplemental Data with this article). Furthermore, we calculated mean values for soil temperature, soil surface temperature and air temperature over the course of the whole experimental period (Table 1). On average, temperatures were intermediate in the garden with 21.2 – 22.6 °C, highest in the greenhouse with 23.9 – 26.1 °C, and lowest in the climate chamber with 20.4 – 21.2 °C (Table 1).

At the start of the experiment at noon, we measured the light intensity using a light meter (Panlux electronic 2, GMC-Instruments, Nürnberg, Germany) in the outdoor garden and greenhouse at 12 spatially distributed pots at the same height 2 cm above the soil. After the end of the experiment, hourly solar radiation data (W•m^{-2}) was extracted for the whole experimental period from a weather station in the outdoor garden (iMetos 1, Pessl Instruments GmbH, Weiz, Austria). To assess, how much radiation the plants received in the outdoor garden, we multiplied the solar radiation data by 0.55 to account for the shading cloth (45 % shading) and converted the data into Photosynthetic Photon Flux Density (PPFD, µmol•m^{-2}•s^{-1}) using the default function *Rg.to.PPFD()* from the *bigleaf* package (Knauer et al., 2018) in R (version 4.0.3, R Core Team, 2020). Fraction of incoming solar irradiance to photosynthetically

active radiation (PAR) was set to 0.5 and the conversion factor was set to 4.6. According to the light intensity measurements at the start of the experiment, the greenhouse received 18.5 % of the light that the outdoor garden plants received. We used this ratio to approximate how much PPFD the plants in the greenhouse received over the course of the experiment by multiplying the PPFD data of the outdoor garden by 0.185. We summed all values per day to calculate the daily light integral (DLI) for each day and then calculated the mean DLI for the whole experimental period. For the climate chamber, we directly measured PPFD at 12 spatially distributed spots at pot height using a PAR sensor (PAR Special sensor SKP 210, Skye Instruments Ltd, Powys, UK). Since the photoperiod was constant in the climate chamber (14h), we multiplied the mean PPFD measurement (297.5 $\mu mol \bullet m^{-2} \bullet s^{-1}$) by the photoperiod (in seconds) to calculate the DLI. The average DLI over the course of the experiment (Table 1) was highest in the garden (20.2 $mol \bullet m^{-2} \bullet d^{-1}$), lowest in the greenhouse (3.7 $mol \bullet m^{-2} \bullet d^{-1}$) and intermediate in the climate chamber (15.0 $mol \bullet m^{-2} \bullet d^{-1}$).

Measurements

During the experiment, we recorded onset of flowering three times per week, because it is an essential trait in the life history of a plant and has often been observed to evolve under changing environmental conditions (Franks, 2015; Pelletier et al., 2009). We regarded a plant as flowering when the first anther was visible. Plants started flowering in August and after three months, most plants had flowered and we measured the rosette diameter as a measure of growth and ability to capture light. We also recorded the number of flowering stems as a proxy for reproductive success. For each individual, we measured the chlorophyll content of four randomly selected healthy and fully developed leaves in SPAD units using a chlorophyll meter (SPAD-502 Plus, Konica Minolta, Neu-Isenburg, Germany). We measured leaf area of three randomly selected healthy and fully developed leaves per plant with the smartphone application “easy leaf area free” (Easlon and Bloom, 2014). These leaves were dried in a dry oven at 60 °C for three days and then weighed together at a fine scale (CPA225D-0CE, e = 1 mg, Sartorius AG, Göttingen, Germany). At the same day, we harvested flower heads and flowering stems as reproductive biomass and leaves as vegetative biomass and dried these using the same procedure. This allows us to detect whether plants invest their resources into vegetative growth or rather into reproductive structures. In order to

investigate responses in leaf anatomy, we calculated specific leaf area (SLA) by dividing the combined leaf area of the three selected leaves by their dry weight and calculated the leaf dry matter content (LDMC) by dividing the dry weight by the fresh weight. The weight of the three selected leaves was added to the vegetative biomass.

Data analysis

All statistical analyses were performed using R (version 4.0.3, R Core Team, 2020). We used linear mixed effects models implemented in the *lme4* package (Bates et al., 2015) with temporal origin, generation, and environment as fixed factor, as well as their two- and three-way interactions. Maternal line was included as random factor and initial size as covariate. Rosette diameter, vegetative biomass, SLA, LDMC, onset of flowering, reproductive biomass, number of flowering stems, and the SPAD measurements were used as response variables, and we applied appropriate transformations to these variables when necessary to improve normality and heteroscedasticity of model residuals (Table 2). All linear models were analysed using the function *Anova()*, and *P* values (Appendix S2) were adjusted for multiple testing with the method of False Discovery Rate (Benjamini and Hochberg, 1995) using the *fuzzySim* package (Barbosa, 2015). Tukey post-hoc tests were applied using the *emmeans* package (Lenth, 2021) whenever an explanatory factor with more than two levels was significant.

Results

The growth facility significantly affected all measured traits (Table 2). In the outside garden, plants had the lowest rosette diameter, and SLA, while those traits were highest in the greenhouse and intermediate in the climate chamber (Fig. 2AC). In contrast, vegetative biomass and LDMC were highest in the climate chamber followed by the garden, and lowest in the greenhouse (Fig. 2BD). A similar pattern can be observed in the onset of flowering (Fig. 3A): plants in the greenhouse and garden flowered at a similar time, but the onset of flowering of plants in the climate chamber was approximately 4 days delayed. The reproductive biomass, the number of flowering stems and SPAD values (Fig. 3BCD) were highest in the garden, intermediate in the climate chamber and markedly low in the greenhouse.

The temporal origin significantly affected vegetative biomass, LDMC, and onset of flowering and we found significant interactions with the growth facility (Origin × Facility) in rosette diameter and onset of flowering (Table 2). Descendants had generally 13 % higher vegetative biomass (Appendix S3A) and 8 % higher LDMC (Appendix S3B) compared to ancestors, irrespective of the growth facility or intermediate generation. Regarding the rosette diameter however, descendants had 11 % higher rosette diameter (2.1 cm) than ancestors in the greenhouse, while values were similar in the garden and in the climate chamber (Fig. 4A). Descendants also flowered 2.6 days later in the climate chamber than ancestors but flowered at similar times in the garden and greenhouse (Fig. 4B).

The number of intermediate generations did not significantly affect any measured trait consistently across growth facilities, but we found significant interactions with the growth facility (Gen × Facility) in rosette diameter and SPAD values (Table 1). Although we did not find significant differences between the F1 and F2 generations within growth facilities for rosette diameter and SPAD values using Tukey post-hoc tests, the F2 had 11 % larger rosettes than the F1 both in the greenhouse and the climate chamber, while the F1 and F2 in the garden were similar (Fig. 4C). Concerning SPAD, the F1 generation showed 3 – 6 % larger values than F2 in the garden and climate chamber, while F1 and F2 did not differ in the greenhouse (Fig. 4D). We found no significant interactions between temporal origin and intermediate generation (Origin × Gen) and no significant three-way-interactions (Origin × Gen × Facility) in the measured traits (Table 2).

Discussion

We studied the effects of three growth facilities and one *vs* two intermediate generations on the phenotypic expression of ancestor and descendant genotypes of a single population of *Leontodon hispidus*. We found very strong phenotypic differences among the three growth facilities. Furthermore, we found significant temporal origin × facility interactions in two traits, indicating that the choice of the growth facility can affect detectability of phenotypic differences. Finally, we did not find differences between intermediate generations within the growth facilities, suggesting that there is

no need for multiple intermediate generations to sufficiently reduce parental and storage effects for this species.

Differences in growth facilities

Plant responses in the different growth facilities varied greatly from each other and the observed patterns were trait specific. The outdoor garden proved to be the experimental environment where plants were most successful in their reproductive ability, as they had the most flowering stems and the highest reproductive biomass. Plants in the garden also had low SLA and high LDMC, which was probably caused by the high light availability present at that growth facilities (Table 1). It has been well established that light irradiance correlates negatively with SLA and positively with LDMC (Anten, 2005; Poorter et al., 2019). Plants in the garden also had the highest chlorophyll content (SPAD values) which could be the reason for the greater reproductive success. Previous studies also showed that leaf traits can strongly respond to water availability, with increasing dryness leading to decreasing SLA and increasing LDMC (Jung et al., 2014; Poorter et al., 2009; Vitra et al., 2019; Volaire, 2008; Wellstein et al., 2017). Accordingly, pots in the garden had the lowest soil moisture content (Fig. 1B) and high variability in soil moisture, which was caused by the exposure to natural rain events, high temperature fluctuations (Fig. 1A), and potentially higher evaporation due to wind exposure.

In the greenhouse, plants grew a large rosette diameter, had high SLA, and low LDMC. This response is in line with a strategy to increase surface area to improve light capture in low-light environments (Poorter et al., 2019). Indeed, the greenhouse, where we expected more favourable conditions than the outside garden, had the lowest light availability (Table 2) due to shading by greenhouse structural elements, but also very high temperatures (Fig. 1A). These high temperatures are also likely to contribute to the high SLA, as they facilitate cell expansion and thus, reduce leaf density and number of cell layers (Atkin et al., 1996; Poorter et al., 2009). The number of flowering stems, reproductive biomass and SPAD values were very low, indicating that even with the adjustments in leaf anatomy, the plants in the greenhouse had the lowest performance and were least successful.

Plants growing in the climate chamber had intermediate rosette diameter, SLA, SPAD values, and reproductive traits, which correlates well with the intermediate light availability (Table 1). Interestingly, plants produced the most vegetative biomass in the climate chamber out of all growth facilities. The very controlled and stable conditions might support fast vegetative growth in the climate chamber without compromising reproductive investment.

The comparisons showed significant differences among growth facilities. The main drivers of these patterns are most likely the differences in light availability and temperature, but also the variability of environmental factors could have a significant impact (Hamann et al., 2021). We expected that the garden would be the least favourable environment due to higher temperatures and stronger environmental fluctuations, leading to decreased growth and fitness of plants. However, our results indicate the opposite, which is probably due to the much higher light availability in outdoor gardens in summer compared to the greenhouse and climate chamber. Also, the garden conditions are much closer to natural conditions to which plants from the field are expected to be adapted (Lascoux et al., 2016).

Reproducibility among growth facilities

Although it can be expected that different growth facilities cause plants to differ in their overall performance, it may also be assumed that origin and treatment effects would show qualitatively similar results across environments. Under this assumption, if a common-environment experiment would be performed in a single environment – as in most studies – the expected patterns would also be observed irrespective of this environment. Two alternative scenarios are, however, possible. First, origin or treatment effects may be observed only under specific environmental conditions and not in others. This would imply that an experiment may not always reveal the expected patterns. Second, specific origin or treatment effects may be observed under the chosen experimental conditions, but contrasting origin or treatment effects could have been observed when alternative experimental conditions would have been chosen. A consequence would be that contrasting conclusions could have been drawn, depending on the experimental environment.

In our study, descendants consistently had higher vegetative biomass and higher LDMC compared to ancestors. These results are in line with the previous findings that this population of *L. hispidus* evolved faster growth in recent decades (Karitter et al., 2024), which were observed in the greenhouse in autumn. Furthermore, high LDMC has been shown to increase drought survival chances (Bongers et al., 2017; De La Riva et al., 2017). LDMC correlates well with strong cell walls and may be beneficial to maintain turgor under drought conditions (Monson and Smith, 1982). Therefore, high LDMC could have evolved in this population through selection by increasing drought events caused by climate change (IPCC, 2018). The phenotypic differences between ancestors and descendants of these two traits were consistent throughout the experimental environments, since we were able to reproduce them in the outdoor garden, greenhouse and in the climate chamber. In contrast, we found interactions of temporal origin with the experimental environment for rosette diameter and onset of flowering. Descendants had a larger rosette diameter in the greenhouse compared to ancestors, but temporal origins did not differ in any other environment. Given that the most prominent distinction of greenhouse was its low light irradiance, increasing rosette diameter may be a good strategy to increase the surface area of the leaves to capture more light. This interaction may be explained by evolution under more shaded conditions, which could have been caused by increased competition during the recent decades exacerbated by climate change (Parmesan and Hanley, 2015). At the collection site, we observed that *L. hispidus* naturally competes with grasses such as *Brachypodium pinnatum* and *Bromus erectus*, which can substantially shade this rosette species. Combined with high nutrient depositions from the atmosphere (Bobbink et al., 2010; Galloway et al., 2008; Newman, 1995) and surrounding agriculture, *L. hispidus* might have faced strong selection pressure through high competition and may have adapted its ability to plastically respond to increasingly shaded conditions (Karitter et al., 2023). Hence, the response to high shading of the descendants was not triggered in the other environments, but this explanation needs further testing by additional experiments that include shading treatments.

We also observed later onset of flowering of descendants compared to ancestors exclusively in the climate chamber. Plants flowered generally later in the climate chamber compared to the other environments, but descendants delayed their

onset of flowering substantially more than ancestors. In another resurrection study on the same population conducted in a greenhouse, descendants also flowered later than ancestors (Rauschkolb, et al., 2022), which was explained by the introduction of sheep grazing in 2007, forcing plants to flower later to escape the grazing pressure. We could not reproduce this result in the greenhouse used in this experiment and also not in the outside garden. Reason for that may be moderately different conditions in the greenhouse and also different timing of the experiment. Therefore, our results also indicate that the experimental environments used in this study provide different environmental cues for the onset of flowering. The onset of flowering is strongly dependent on environmental cues and plants can accelerate or delay it depending on local conditions in order to guarantee seed production for the next generation (Coupland, 1995). Generally, plants tend to flower later at colder temperatures (Capovilla et al., 2015), and, indeed, the average temperature was significantly lower in the climate chamber compared to the garden or greenhouse. Furthermore, the photoperiod differed in the climate chamber with approximately 2.5 h less light per day compared to the garden. Finally, the climate chamber provides the most stable conditions compared to the other environments. Descendants showed a different phenology under the shorter photoperiod, more benign temperatures and more stable conditions in the climate chamber. High variability in environmental variables in general, and even more so under climate change, is the norm under natural conditions, and perennials such as *L. hispidus* might take advantage of a stable period to grow vegetatively in order to ensure survival and increase future reproductive output, thus delaying the onset of flowering time (Tun et al., 2021). However, following this explanation, we would also expect higher vegetative biomass of descendants in the climate chamber, which was not the case. If grazing at annually recurring times selected for later flowering plants, as suggested by Rauschkolb and colleagues (2022), then the underlying process causing delayed flowering time might be through shifts in photoperiod requirement.

The majority of resurrection studies investigate the evolutionary responses of plant populations only in a single growth facility. As our study shows, depending on the trait, phenotypic differences are not guaranteed to be detected in a given experimental environment (i.e., growth facility), even if genetic differences are present. Especially when investigating evolution of plant populations, having the experimental conditions

as close to their natural habitat as possible is desirable to detect evolution to the contemporary environmental conditions. With this approach, we can study the phenotypes that would occur in the field under natural conditions. However, creating deviating or even stressful environmental conditions may reveal genetic differences that are only expressed during a stressful period. The selection that caused genetic differences between ancestors and descendants could have been applied on plasticity under stressful conditions, which can only be observed under those stressful conditions. But care should be taken, because extreme conditions may also elicit responses that are not being expressed under natural extreme conditions (Ghalambor et al., 2007). When choosing between the three growth facilities we tested in this study, the best choice depends on the research aims. The garden seems to be the best option to mimic natural conditions, because of natural light, low spatial heterogeneity and contemporary weather conditions. The greenhouse seems to be the poorest choice for natural conditions, because of low light intensity and high temperatures, although it is the most used in resurrection studies (e.g., Anstett et al., 2021; Franks et al., 2007; Gay et al., 2022; Hamann et al., 2018; Kuester et al., 2016; Lambrecht et al., 2020; Nevo et al., 2012; O'Hara et al., 2021; Sultan et al., 2013; Vtipil & Sheth, 2020). Chiang and colleagues (2021) showed that environmental fluctuations can affect the phenotypic expression of multiple traits and are important to study natural-like plant growth. Here, climate chambers present an intriguing option going forward if they are programmed very closely to field conditions. Using climate or weather data from the collection sites, one could program the average temperatures, moisture, light spectrum and their daily to seasonal variability to have the conditions very close to the field, while still having a high level of control (Poorter et al., 2016). This idea has already been successfully applied by Heuermann and colleagues (2023) who managed to simulate whole seasons in a reproducible manner in their specialized indoor growth facility. Additionally, extreme events can be modelled (e.g., heatwaves) as treatments to further investigate if differences are also expressed under such conditions, especially if these conditions are potential selection agents. Very controlled and homogenous conditions on the other hand, can be useful if only genetic differences per se are of interest and not how they relate to the actual natural conditions. Especially low temporal variation in experimental conditions is important to provide similar conditions throughout all ontogenetic stages, and to avoid interactions of ontology and the environmental conditions. Furthermore, it is possible to infer adaptive traits in the field

from genotypes grown indoors by using modelling of the environmental conditions (Bouidghaghen et al., 2023).

Intermediate generations

We found no differences between the two intermediate generations used in this study within each of the growth facilities, indicating either that no significant parental effects were present beforehand at all or that they were removed after the first intermediate generation. The magnitude and nature of parental effects can be strongly dependent on the environmental stresses experienced by the parents, as Latzel and colleagues (2023) showed that theses stresses can affect the fitness of the offspring by up to 35 % in *Arabidopsis thaliana*. It is likely that environmental stresses differed between ancestors and descendants of our study population, as climate change increased the frequency and duration of droughts and heatwaves (IPCC, 2018). However, if there were any detectable parental effects, these have been eliminated in the first intermediate generation, which has also been found in other studies (Agrawal, 2002; Gianoli, 2002). Since we did not have seed material of the originally collected sample – i.e., before the intermediate generations –, we cannot quantify how much the first intermediate generation reduced parental and storage effects. Thus, we cannot know whether there were detectable parental effects in the first place. Theoretically, long-term storage of seeds may cause carry-over effects into the F1-generation in suboptimal storage conditions, with resurrected plants having lower fitness due to the storage and producing lower quality seeds (Gebeyehu, 2020). The seed material used in this study was stored at -20°C after drying at 15% RH which are optimal conditions to ensure viability for several decades (Solberg et al., 2020). The implications of potential carry-over effects can of course be very dependent on the storage condition and storage length, and other species might be affected more than *L. hispidus*. Thus, multispecies experiments are needed to advance our understanding of parental and storage effects and to make informed choices regarding the amount of required intermediate generations for a given species.

Conclusions

Our study shows that the choice of the growth facility in common-environment experiments can potentially impact the expression of phenotypic differences among genotypes, thereby affecting the conclusions. Thus, studying evolution of plant populations in only a single environment might result in incomplete or even deficient interpretations for some traits. Hence, it is important to carefully choose the growth facility or even use multiple facilities. Outdoor garden experiments might be a good and simple option with regard to studying rapid evolution as plants will experience more natural conditions and the contemporary climate which they are expected to have evolved to. However, if environmental variables from the population are well known, using climate chambers might be a good alternative with a high level of control and detailed programming to encompass realistic natural conditions as well as less or more extreme conditions that may occur under natural conditions, e.g., as additional treatments. Finally, growing a second intermediate generation rather than only one intermediate generation did not impact the genetic differences of ancestors and descendants within growth facilities, suggesting that only one intermediate generation would be sufficient to reduce detectable parental and storage effects, if there were any in the first place. Overall, future studies should be aware of implications regarding reproducibility and wisely choose the experimental conditions.

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Data archiving

The data that support the findings of this study will be made available at Dryad after this manuscript is accepted for publication.

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Tables and figures

Table 1. Mean values and standard errors of environmental variables over the whole experimental period for all growth facilities.

Growth facility	Temperature [°C]			Daily light integral
	Soil (5cm)	Soil surface	Air	[mol•m^{-2}•d^{-1}]
Garden	21.2 ± 0.2	22.2 ± 0.3	22.6 ± 0.3	20.2 ± 0.8
Greenhouse	23.9 ± 0.2	25.5 ± 0.2	26.1 ± 0.2	3.7 ± 0.1
Climate chamber	20.4 ± 0.05	21.2 ± 0.03	20.7 ± 0.04	15.0 ± 1.0

Table 2. Results of the statistical models testing the effects of temporal origin (ancestors, descendants), intermediate generation (F1, F2), growth facility (garden, greenhouse, climate chamber) and their interactions on the response variables (y) rosette diameter, vegetative biomass, specific leaf area (SLA), leaf dry matter content (LDMC), onset of flowering, reproductive biomass, number of stems and SPAD measurements. We used linear mixed effects models with initial size as covariate and maternal line as random factor followed by ANOVA's. Response variables were transformed if needed to fulfil parametric assumptions. Shown are degrees of freedom (df), *F* values and adjusted *P* values using False Discovery Rates. Significant p values are shown with asterisks (*: $P \leq 0.05$; ***: $P \leq 0.001$) and marginally significant values are shown in bold.

		Rosette diameter		**Vegetative biomass**		**SLA**		**LDMC**		**Onset of flowering**		**Reproductive biomass**		**Number of stems**		**SPAD**	
	df	*F* value	*P* value adj	*F* value	*P* value adj	*F* value	*P* value adj	*F* value	*P* value adj	*F* value	*P* value adj	*F* value	*P* value adj	*F* value	*P* value adj	*F* value	*P* value adj
Initial size		65.42	***	25.00	***	8.41	*	17.28	***	20.74	***	2.09	0.3	0.14	0.8	0.78	0.6
Origin	1	2.61	0.3	8.55	*	0.18	0.8	9.39	*	7.01	*	0.00	1.0	1.65	0.4	2.90	0.3
Gen	1	2.26	0.3	0.69	0.6	1.70	0.4	0.23	0.8	1.75	0.4	0.18	0.8	0.25	0.8	2.45	0.3
Facility	2	155.48	***	301.09	***	199.31	***	91.46	***	50.73	***	457.85	***	280.86	***	210.71	***
Origin × Gen	1	0.31	0.8	2.78	0.3	0.23	0.8	1.30	0.5	0.71	0.6	0.20	0.8	1.12	0.5	0.95	0.6
Origin × Facility	2	3.91	**0.067**	1.55	0.4	0.52	0.8	0.53	0.8	5.57	*	2.07	0.3	2.71	0.2	0.11	0.9
Gen × Facility	2	4.77	*	0.39	0.8	1.00	0.6	0.68	0.7	0.41	0.8	0.09	0.9	1.98	0.3	4.57	*
Origin × Gen × Facility	2	0.10	0.9	0.32	0.8	0.24	0.9	1.52	0.4	0.30	0.8	1.47	0.4	1.75	0.4	0.20	0.9
Transformation		$(x)^{1.5}$		(x)		log(x)		sqrt(x)		$(x)^2$		$(x)^{0.3}$		$(x)^{0.9}$		(x)	

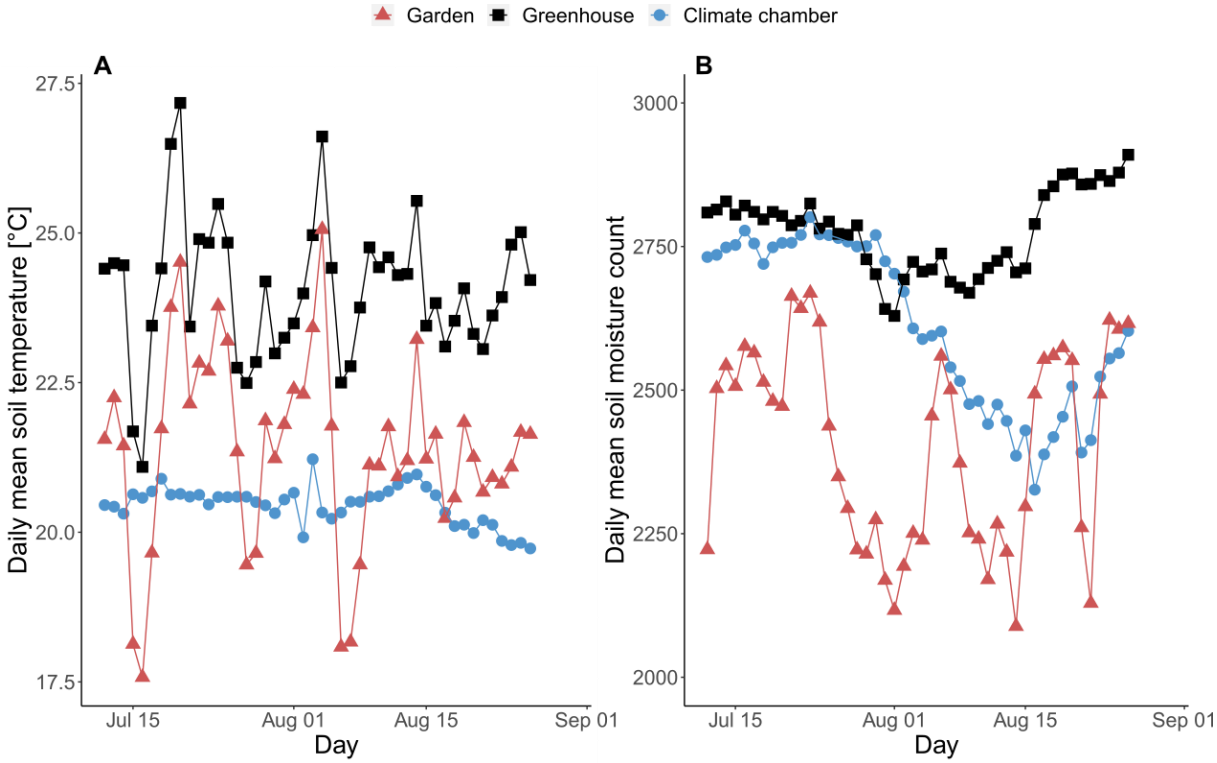


Figure 1. Daily mean soil temperature (A) and daily mean soil moisture count (B) of four random pots grown in different growth facilities. The growth facilities are garden (red triangles), greenhouse (black squares), and climate chamber (blue circles).

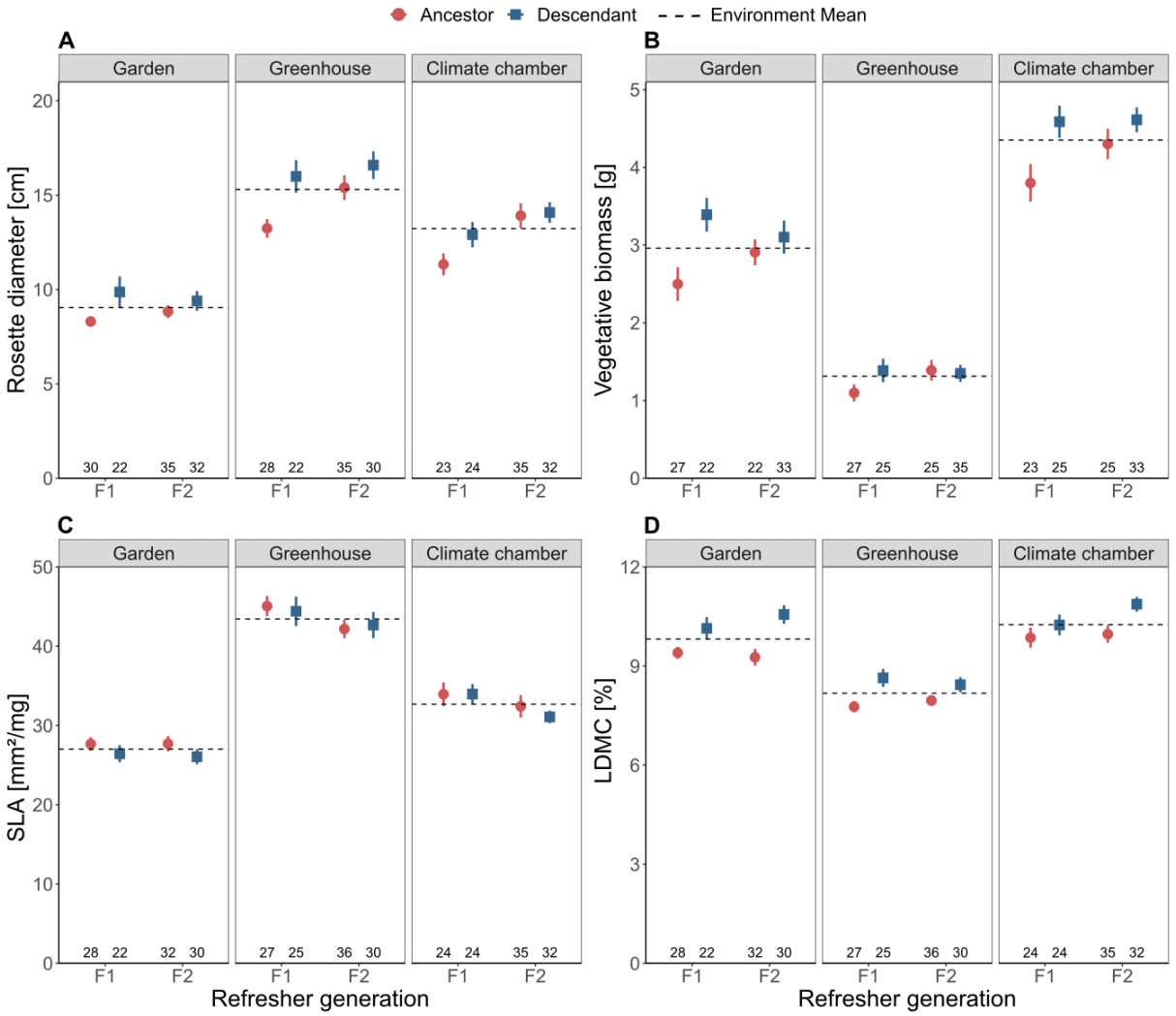


Figure 2. Rosette diameter (A), vegetative biomass (B), specific leaf area (C) and leaf dry matter content (D) of ancestors (blue) and descendants (red) after one intermediate generation (F1) and two intermediate generations (F2) grown in different growth facilities (garden, greenhouse, climate chamber). Shown are means and standard errors. The dotted line represents the overall mean value in each environment. Sample sizes are given at the bottom of the graph below their respective data point.

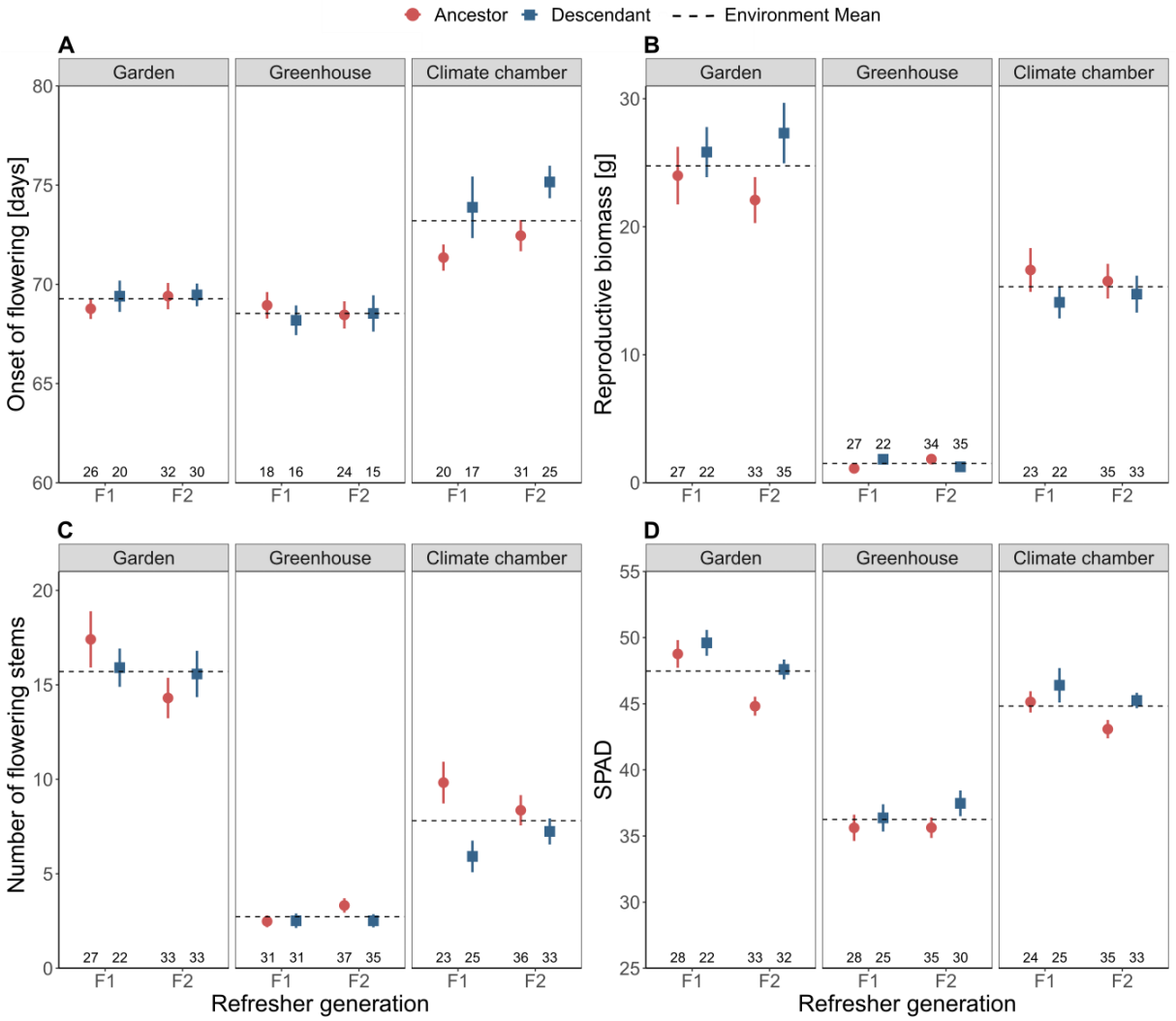


Figure 3. Onset of flowering (A), reproductive biomass (B), number of flowering stems (C) and SPAD values (D) of ancestors (blue) and descendants (red) after one intermediate generation (F1) and two intermediate generations (F2) grown in different growth facilities (garden, climate chamber, greenhouse). Shown are means and standard errors. The dotted line represents the overall mean value in each environment. Sample sizes are given at the bottom of the graph below their respective data point.

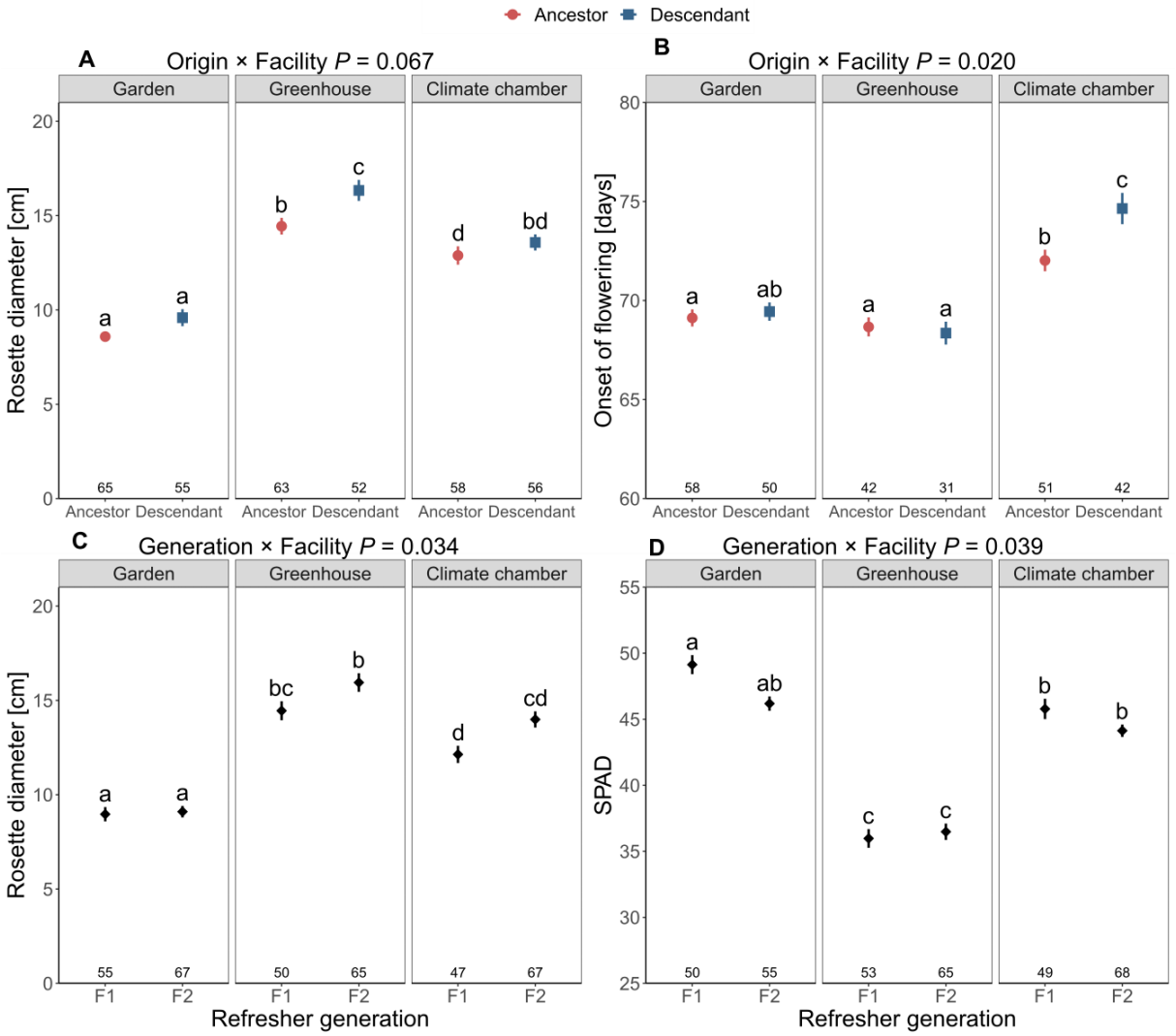
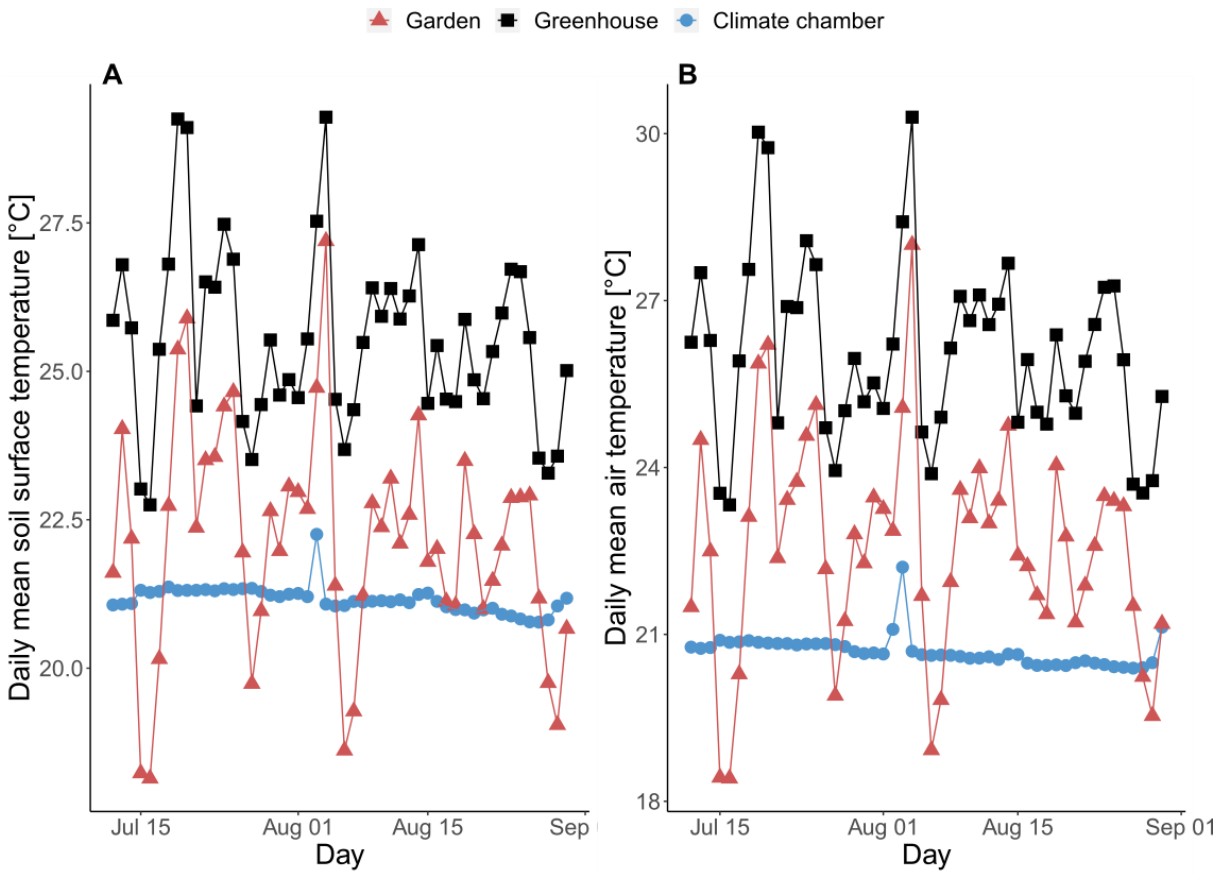


Figure 4. Rosette diameter (A) and onset of flowering (B) of ancestors (red) and descendants (blue) in the different environments (significant Origin × Facility effect). Rosette diameter (C) and SPAD values (D) of F1 and F2 intermediate generations in different facilities (significant Generation × Facility effect). Shown are means and standard errors. Sample sizes are given at the bottom of the graph below their respective data point.

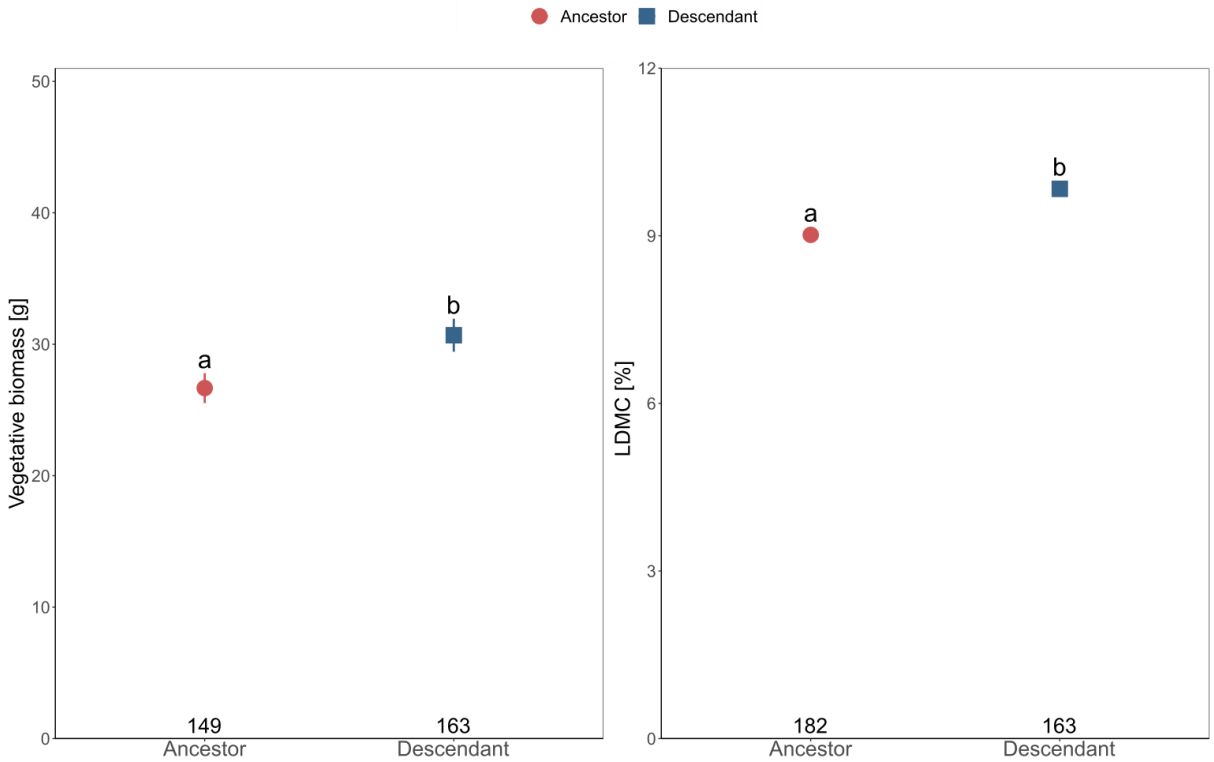
Supporting information – Chapter III



Appendix S1. Daily mean soil surface temperature (A) and daily mean air temperature (B) of four random pots grown in different growth facilities. The growth facilities are garden (red triangles), greenhouse (black squares), and climate chamber (blue circles).

Appendix S2. Results before adjustments of the statistical models testing the effects of temporal origin (ancestors, descendants), intermediate generation (F1, F2), growth facility (garden, greenhouse, climate chamber) and their interactions on the response variables (y) rosette diameter, vegetative biomass, specific leaf area (SLA), leaf dry matter content (LDMC), onset of flowering, reproductive biomass, number of stems and SPAD measurements. We used linear mixed effects models with initial size as covariate and maternal line as random factor followed by ANOVA's. Significant values ($P < 0.05$) are shown in bold.

		Rosette diameter	**Vegetative biomass**	**SLA**	**LDMC**	**Onset of flowering**	**Reproductive biomass**	**Number of stems**	**SPAD**
	df	*P* value	*P* value	*P* value	*P* value	*P* value	*P* value	*P* value	*P* value
Initial size		**<0.001**	**<0.001**	**0.004**	**<0.001**	**<0.001**	0.15	0.708	0.379
Origin	1	0.118	**0.007**	0.676	**0.005**	**0.014**	0.969	0.210	0.101
Gen	1	0.145	0.414	0.204	0.639	0.198	0.673	0.623	0.130
Env	2	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Origin × Gen	1	0.584	0.106	0.633	0.264	0.408	0.659	0.299	0.339
Origin × Env	2	**0.021**	0.214	0.593	0.590	**0.004**	0.128	0.068	0.899
Gen × Env	2	**0.009**	0.678	0.371	0.507	0.661	0.912	0.140	**0.011**
Origin × Gen × Env	2	0.902	0.728	0.785	0.219	0.740	0.232	0.176	0.817



Appendix S3. Vegetative biomass (A) and LDMC (B) of ancestors and descendants (significant Origin effect). Shown are means and standard errors. Standard errors of LDMC are too small to be visible. Sample sizes are given at the bottom of the graph below their respective data point.

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