Back to the Future –
Seed Banks as a Tool to Investigate Recent Adaptation to Global Change

Dissertation

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Erklärung

Zulassung als Doktorand im Sinne von § 4 Abs. 1 der Promotionsordnung vom 24. April 2015 erfolgte am 23. April 2018. Diese Dissertation wurde im Sinne von §6 von Prof. Dr. Johannes Fredericus Scheepens und Prof. Dr. Oliver Bossdorf betreut.

Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass diese Dissertation von mir selbstständig – abgesehen von der Beratung und Hilfe meiner Betreuer – und ohne unerlaubte Hilfsmittel erarbeitet wurde. Andere als die angegebenen Quellen und Hilfsmittel wurden nicht benutzt und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen wurden als solche kenntlich gemacht.

Diese Dissertation wurde an keiner anderen Prüfungsbehörde vorgelegt.

Tübingen, 12.01.2022

…… (Robert Rauschkolb)

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1. Gutachter: Prof. Dr. Johannes Fredericus Scheepens
2. Gutachterin: Prof. Dr. Katharina Förster

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Declaration of author contributions

The thesis entitled ”Back to the future - Seed Banks as a Tool to Investigate Recent Adaptation to Global Change” is based on the work I did during my PhD at the University of Tübingen, supervised by Prof. Dr. Johannes Fredericus Scheepens and Prof. Dr. Oliver Bossdorf. Chapter I – III in this thesis include three independent scientific manuscripts. Each chapter contains co-authorship, and is or will be published. The contribution of the authors for each chapter is stated as following:

Chapter I
Robert Rauschkolb, Walter Durka, Sandrine Godefroid, Lara Dixon, Oliver Bossdorf, Andreas Ensslin, JF Scheepens: *Parallel evolution of advanced flowering onset across multiple European plant species in response to increased aridity over the last decades*

Status in publication process: in preparation

RR, AE and JFS conceived the experiment. RR, AE and JFS designed the experiment. RR, SG, and LD conducted fieldwork and RR performed the experiment. RR collected data and performed data analysis with input from OB, AE and JFS. WD performed the molecular analyses. RR wrote the manuscript with input from all co-authors.

Chapter II
Robert Rauschkolb, Lisa Henres, Caroline Lou, Sandrine Godefroid, Lara Dixon, Walter Durka, Oliver Bossdorf, Andreas Ensslin, JF Scheepens: *Historical comparisons show evolutionary changes in drought responses in European plant species after two decades of climate change*

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RR, AE and JFS conceived the experiment. RR, LH, CL, AE and JFS designed the experiment. RR, LH, SG and LD conducted fieldwork and RR, LH, CL performed the experiment. RR, LH und CL collected data and performed data analysis with input from OB, AE and JFS. WD performed the molecular analyses. RR wrote the manuscript with input from all co-authors.

Chapter III
Robert Rauschkolb, Zixin Li, Sandrine Godefroid, Lara Dixon, Walter Durka, Maria Májeková, Oliver Bossdorf, Andreas Ensslin, JF Scheepens: *Evolution of drought strategies and herbivore resistance after two decades of climate change in European plants*

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RR, AE and JFS conceived the experiment. RR, ZL, AE and JFS designed the experiment with input from MM. RR, SG and LD conducted fieldwork and RR and ZL performed the experiment. RR and ZL collected data and performed data analysis with input from OB, AE and JFS. WD performed the molecular analyses. RR wrote the manuscript with input from all co-authors.

Summary

Climate change poses challenges for all ecosystems. Plants, as sessile organisms, are particularly vulnerable to shifts in their environmental conditions and are at high risk for local extinction. To avoid extinction and to cope with novel conditions, plant populations can respond through adaptive evolution, which sometimes only requires a few generations to occur. Scientists are using several methods to study how climate change leads to shifts in plant traits. For example, there are studies with herbaria specimens and/or old data collections that include many different species, which help to detect general patterns of trait shifts (e.g. flowering onset). However, such observational studies do not differentiate between plastic responses of plants and the possibility of adaptive evolution. In order to discern between these possibilities, **"forward-in-time"** resurrection experiments, where ancestors of a single population are revived and compared to their descendants, can be used. Thus, resurrection experiments are useful for detecting the rate of phenotypic evolution, examining potential trade-offs with co-occurring environmental conditions, and learning about evolutionary rescue, restoration and conservation. Although the number of publications using the **"forward-in-time"** resurrection approach is continuously increasing, the published studies often focus only on single species and investigate only one or a few populations. In order to broaden the possibilities of studying the evolution of plant traits in response to climate change, I aimed with this work to turn the **"forward-in-time"** resurrection approach into a **"back-in-time"** mode, by comparing ancestral seed material stored in seed banks with their contemporary populations.

To gain access to ancestral seed material, I established collaborations with five European seed banks and eventually conducted several common garden greenhouse experiments with 18 plant species, always comparing ancestors with their descendants. In **Chapter I**, I studied 13 species and investigated differences between ancestors and descendants with regard to phenology (flowering onset) and early growth. Here, I further related climatic data to trait differentiations of the temporal origins. In **Chapters II** and **III**, I used watering treatments as a proxy for climate change, which also enables testing for adaptations to drought. In **Chapter II**, using multiple species detecting general patterns, I focused on trait differentiation in early life stages in response to drought. Whereas the usage of only four species in **Chapter III** allowed me to increase precision and to study interactions of responses to drought with co-occurring herbivory. Furthermore, I disentangled selective from random forces driving recent trait changes by performing Q_{ST}- F_{ST} comparisons in this study.

Across the three chapters of this thesis, I found evidence that the descendants advanced

their life cycles through rapid growth and advanced flowering. As the populations originated from regions where drought frequencies and intensities have increased during the last decades, it is comprehensible that the observed trait shifts may reflect escape strategies to avoid drought stress in summer. As the observed patterns were consistent across multiple-species, I hypothesise that trait differentiations between ancestors and descendants are the result of selective instead of random evolutionary processes. This assumption is further confirmed by my Q_{ST}-F_{ST} comparisons in **Chapter III,** which shows that flowering onset was under directional selection.

Besides detecting phenotypic trait shifts that indicate rapid evolution of European plant populations during the last decades, one goal of this thesis was to establish the **"back-in-time"** mode for resurrection studies using ancestral seed material stored in seed banks. After performing my experiments and minimising apparent uncertainties of this approach, I am confident that seed bank collections are untapped resources that could enable a variety of future studies, especially with multiple species, to investigate evolutionary changes of plant traits in response to climate change.

Zusammenfassung

Der Klimawandel stellt alle Ökosysteme vor enorme Herausforderungen. Insbesondere Pflanzen sind als sessile Organismen für Veränderungen in ihrer Umwelt anfällig. Um mit sich ändernden Bedingungen zurecht zu kommen, können Pflanzen sich evolutionär anpassen, was wiederum teilweise nur weniger Generationen bedarf. In der Forschung werden unterschiedliche Methoden verwendet, um zu untersuchen, wie der Klimawandel zu Verschiebungen in Pflanzenmerkmalen (z.B. Blühzeitpunkt) führt. Ein Beispiel hierfür sind Studien mit Herbariumsbelegen und/oder historischen Datenaufzeichnungen, welche viele unterschiedliche Pflanzenarten beinhalten können. Bei dieser Art von Untersuchung kann jedoch nicht zwischen plastischen Reaktionen der Pflanzen und evolutionären Anpassungen unterschieden werden. **"Forward-in-time resurrection studies"**, bei welchen man lebende Pflanzen von Vor- und Nachfahren einer Population miteinander vergleicht, machen diese Unterscheidung möglich. So bilden "**resurrection studies"** eine zuverlässige Methode, um die Evolution in Phänotypen und mögliche Trade-Offs mit anderen Umweltfaktoren zu bestimmen. Des weiteren kann durch ihre Anwendung Wissen für die Wiederansiedlung und den Schutz von Arten generiert werden. Obwohl in den letzten Jahren die Anzahl an Publikationen, welche den **"resurrection approach"** genutzt haben, stetig zugenommen hat, untersuchen diese Studien meistens nur einzelne Arten und berücksichtigen hierbei nur eine oder wenige Populationen. Ein Ziel der vorliegenden Arbeit ist es die Möglichkeiten, wie man evolutionäre Anpassungen in Pflanzen untersuchen kann, weiter auszuschöpfen. Dabei wird so vorgegangen, dass der klassische **"forward-in-time"** Modus zu einem **"back-in-time"** Modus verändert wird, indem bereits gesammelte und in Samenbanken gelagerte Samen, als Vorfahren für die Experimente genutzt und mit neu gesammelten Nachfahren der gleichen Populationen verglichen werden.

Den Zugang zu den Samen der Vorfahren hat eine Zusammenarbeit mit fünf europäischen Samenbanken ermöglicht, sodass am Ende unterschiedliche Gewächshausexperimente mit insgesamt 18 Arten durchgeführt werden konnten. **Kapitel I** beinhaltet eine Untersuchung von insgesamt 13 Pflanzenarten, wobei Unterschiede zwischen Vor- und Nachfahren in Blühzeitpunkt und in Wachstum im Fokus stehen. Zusätzlich werden diese Ergebnisse mit lokalen Klimadaten der letzten Jahrzehnte in Zusammenhang gesetzt. **Kapitel II** und **III** legen das Augenmerk auf Bewässerungsbehandlungen zur Simulierung des Klimawandels. Dadurch kann bestimmt werden inwiefern die untersuchten Pflanzenpopulationen an Trockenheit angepasst sind. **Kapitel II** beleuchtet Merkmalsunterschiede zwischen Vor- und Nachfahren von 13 Arten unter Trockenheitsbedingungen in frühen Lebensstadien. Da im Unterschied dazu in **Kapitel III** nur vier Arten Gegenstand der Untersuchung sind, kann die Genauigkeit der Analysen erhöht und weitere

Merkmale, sowie den Einfluss der Kombination aus Trockenheit und Insektenfraß getestet werden. Darüber hinaus wurde mit Hilfe von Q_{ST}- F_{ST} Analysen auch der Einfluss von selektiven und zufälligen Faktoren auf die Evolution der Merkmale untersucht.

Im Rahmen der vorliegenden Arbeit konnten Hinweise dafür gefunden werden, dass die Nachfahren ihre Lebenszyklen durch schnelles Wachstum und frühere Blühzeitpunkte zeitlich nach vorne gelegt haben. Da die Populationen aus Regionen stammen, in welchen in den letzten Jahrzehnten die Häufigkeit und Intensität von Trockenheit zugenommen hat, ist zu vermuten, dass die beobachteten Merkmalsveränderungen dazu dienen Trockenstress im Sommer zu vermeiden. Die Ergebnisse sind über viele Pflanzenarten hinweg konsistent, so ist davon auszugehen, dass sie Folge von selektiven, nicht zufälligen evolutionären Prozessen sind. Die Ergebnisse der Q_{ST}- F_{ST} Analysen in **Kapitel III** konnten diese Vermutung teilweise bestätigen.

Neben der Untersuchung von Merkmalsveränderungen als Antwort auf den Klimawandel, welche auf eine rasche Evolution der Pflanzenpopulationen in Europa hindeuten, ist ein Anliegen dieser Arbeit den **“back-in-time”** Modus innerhalb der **“resurrection Studien”** zu etablieren. Die durchgeführten Experimente machen deutlich, dass Samen aus Samenbanken (bei einer Reduzierung offensichtlicher Ungenauigkeiten) eine bisher ungenutzte genetische Ressource für Experimente darstellen. Eine Vielzahl von zukünftigen Multi-Arten-Studien, in welchen evolutionäre Anpassungen von Pflanzen als Antwort auf den Klimawandel untersucht werden, könnte durch ihre Verwendung ermöglicht werden.

General Introduction

Plant adaptations to climate change

Since humans started to transition from being “hunter-gatherers“ to “settled farmers“ 10.000–15.000 years ago (Solheim 1972), they have had increasingly stronger impacts on atmospheric, geological and biological processes. Especially during the 20th century, environmental conditions changed rapidly all over the world (Jump and Peñuelas 2005; Steffen *et al.* 2015; Waters *et al.* 2016). In this context, anthropogenic climate change has in particular increased over the last several decades, resulting in higher temperatures (IPCC 2021) and changes in precipitation patterns (Semmler and Jacob 2004; Dore 2005). Warmer temperatures and lower water availability, especially during the growing season in spring and summer, translate into novel and more stressful conditions for plant populations (Anderson *et al.* 2012; Shaw and Etterson 2012; Fleta-Soriano and Munné-Bosch 2016). Thus, plant species are at higher risk of local extinction (Thomas *et al*. 2004; Urban 2015), especially since plants are sessile organisms and therefore more vulnerable to drastic changes to their environmental conditions compared to animals. In order to cope with these changes, plant populations can either (1) track suitable conditions through migration (Davis and Shaw 2001; Parmesan and Yohe 2003; Jump and Peñuelas 2005; Lenoir *et al*. 2008) or respond through (2) adaptive evolution of trait means (Holt 1990; Davis and Shaw 2001; Hoffmann and Sgrò 2011), or through (3) phenotypic plasticity (Sultan 2003; Pigliucci 2005). With respect to adaptation and phenotypic plasticity, three strategies have been identified – “escape”, “avoidance” and “resistance” (Levitt 1987; Barton and Koricheva 2010) – to cope with the increasingly adverse conditions. One specific stressor is water shortage, as a result from climate change. For example, several studies comparing drought adapted with non-drought adapted populations showed that advanced flowering can help plants to escape from droughts in the summer (Franks *et al*. 2007; Kigel *et al.* 2011; Metz *et al*. 2020). In addition, higher investment into roots (Sharp and LeNoble 2002; Martin and Stephens 2006; Villagra and Cavagnaro 2006; Aroca 2012), rooting depth (Padilla and Pugnaire 2007) or reduced aboveground growth (Kusaka *et al*. 2005; Borrell *et al.* 2014) help plants to avoid drought stress as they can increase access to water reservoirs in the soil and reduce evapotranspiration, respectively. Finally, plants can resist drought stress by adjusting their osmotic potential (Kolb and Sperry 1999; Bartlett *et al*. 2014; Májeková *et al*. 2019) or by increasing their water-use-efficiency (WUE, Hatfield *et al.* 2001; Hatfield and Dold 2019).

The above mentioned constitutive changes in traits in response to drought may be adaptive in environments that become generally drier. However, drought events are often periodic

and forecasts for climate change also predict higher climatic variability (IPCC 2013; Kharin *et al.* 2007; Gherardi and Sala 2019). Therefore, changing functional trait values through phenotypic plasticity can be a better strategy than to evolve constitutive changes in trait means (Sultan and Spencer 2002; Alpert and Simms 2002; Gianoli and Valladares 2012). As the breadth of phenotypic plasticity itself is a genetically controlled trait, it may also undergo evolution by natural selection (West-Eberhard 1989; Ackerly *et al*. 2000; Pigliucci 2005; Richards *et al*. 2006).

How to study changes in plants in response to climate change

Several methods can be used to study shifts in plant traits in response to climate change. Here I describe and compare three of them: (1) historical comparisons using herbaria specimen and old data collections, (2) experimental approaches manipulating environmental conditions, (3) the **resurrection approach** (Franks *et al.* 2018), which uses stored seeds to compare revived lineages of ancestral plant populations with their descendants. Through historical comparisons using long-term observations (Fitter and Fitter 2002; Thomas *et al.* 2004) or specimen from herbaria (Panchen *et al.* 2012; DeLeo *et al.* 2019; Lang *et al.* 2019), researchers can travel back in time and investigate whether and to what extent plant species have already responded to climate change. For example, Fitter and Fitter (2002) showed that for 385 British plant species, the average first flowering date had advanced by 4.5 days when comparing observations from 1991–2000 with observations from 1954–1990. Such research is unique as it includes a large number of species and is therefore crucial to understanding the general relationship between climate change and shifts in phenology. However, it remains unclear whether the observed patterns are due to plastic responses or whether plant populations have already adapted to novel environmental conditions through adaptive evolution.

In order to discern between these possibilities, **"forward-in-time"** experimental evolution studies and **"forward-in-time"** resurrection experiments can be used (Franks *et al.* 2018). Since the 1990s the number of studies in which researchers investigated the impact of manipulated environmental conditions on plants and vegetation has increased (Jentsch *et al.* 2007). Such experiments may help to predict future evolutionary shifts in plant traits in response to climate change. However, experiments that use this approach are often limited in duration (< 10 years of observation) and in space, only consider a small number of environmental factors and lack the complexity of natural systems (Leuzinger *et al.* 2011; Kawecki *et al.* 2012). Nevertheless, by using annual species and breeding lines which are adapted to specific environmental conditions, this

approach can be a powerful tool to disentangle plastic responses of plants from adaptive evolutionary processes (Grossman and Rice 2014; Ravenscroft *et al.* 2015; Metz *et al.* 2020). For instance, Metz and colleagues compared populations along a precipitation gradient in the Middle East of *Biscutella didyma*, an annual Brassicaceae, that have experienced artificial watering regimes for 10 years leading to experimental evolution. They found in a common garden experiment that the drought-treated population flowered 3–4 days earlier than the controlled population (Metz *et al.* 2020).

Resurrection experiments using the **"forward-in-time"** mode are similar to experimental evolution studies. Here, ancestors of a single population are revived and compared to their descendants (Elena and Lenski 2003; Franks *et al*. 2018). Franks and colleagues (2018) recommend criteria and processes for performing such investigations using seed material from natural populations. At a specific point in time (T1) seeds of the target population are collected and stored under cool, dry and dark conditions to minimise loss of viability. After several years of natural changes in climate at the site of origin contemporary seeds are collected (T2). To make sure that the ancestral and descendant collections are an unbiased representation of the genetic variation in the studied population, the sample size should be sufficiently large (>30 individuals; Hale *et al.* 2012; Nazareno *et al.* 2017). In addition, seeds should be collected multiple times during the ripening phase to represent the spatial and temporal variability of the population. For standardising both temporal origins (T1) and (T2) and to reduce maternal and storage effects, refresher generations should be grown under common conditions. To reduce the risk of invisible fractions at this point the germination rates for both temporal origins should be high (>75%) or at least equal (Weis 2018). Researchers may use these generations to create genetic lines ("experimental generation") with controlled pollination (e.g. half-sibs within or hybrids between T1 and T2). Afterwards seeds of the "experimental generation" can be used in common garden experiments to detect evolution via phenotypic differences between the lines.

During the last 15 years, an increasing number of studies have used the **"forward-in-time"** resurrection approach to examine evolution of plant populations to climate change and in particular to drought. In line with the strategy to escape from drought in summer by advanced flowering, several studies have shown that descendants flowered earlier than their ancestors (Franks *et al*. 2007; Nevo *et al*. 2012; Vigouroux *et al*. 2011; Thomann *et al*. 2015). Similar to studies using experimental evolution the above-described method directly tests for evolution in plant populations but it represents past responses to climate change and includes all environmental factors and their naturally occurring interactions (Leuzinger *et al.* 2011; Kawecki *et al.* 2012; Franks *et al.* 2018). Resurrection experiments help to detect the rate of phenotypic

evolution and potential trade-offs with co-occurring environmental conditions. They can be used to monitor responses to climate change and gain knowledge for evolutionary rescue, restoration and conservation (Etterson *et al.* 2016; Franks *et al.* 2018). However, such studies are resource intensive and time consuming and they demand planning ahead (Franks *et al.* 2018). Thus, the number of experiments performed is still small. In addition, these experiments often only focus on a single species and investigate one or a few populations.

Seed banks – A untapped resource for climate change research

In order to perform a proper resurrection approach, experiment one has to collect seeds at T1, wait until time passes and climate change occurs, and collect seeds at T2. An alternative to this time-consuming process could be an experiment that compares plants revived from seeds collected in the past with their contemporary descendants from the same population (Everingham *et al.* 2021). Potential resources for ancestral seed material are seed banks, which play an important role in ex-situ conservation of species and genetic richness (Liu *et al.* 2018). In the early days of controlled seed storage, almost 60 years ago, seed banks mainly focused on crop plants to safeguard food supply. However, in the last decades the amount of stored wild accessions increased conspicuously with the aim to conserve threatened as well as commonly occurring wild species and the intraspecific genetic diversity within and among their populations (Wyse 2001; Godefroid *et al.* 2011; Liu *et al.* 2018). The large potential of seed bank collections as a resource for climate change research was demonstrated by Hay and Probert (2013), who listed 1750 seed banks worldwide, and by Godefroid and colleagues (2011), who showed that 29 seed banks in Europe hold 41.928 European/Eurasian accessions. In order to use seed material in experiments along the same lines as the resurrection approach described by Franks and colleagues (2018), the seed collections need to fulfil some general criteria:

1. Sampling locations must be known

To resample descendants of the ancestral population, the original sampling site should be described in an adequate and accurate way. Liu and colleagues (2018) found that 88% of 82.556 collections in the “Millenium Seed Bank“ (MSB, Smith *et al.* 1998) include geo-references, which is common for accessions collected after 2000. Although geo-referenced data would be the most accurate information about the sampling location of the ancestors, descriptions of the site may also be included in the data sheets and these descriptions could be precise enough to relocate the target population even without geo-references. Knowledge from local botanists and nature conservation practitioners could also be helpful to locate previously sampled locations. For

instance, they could know about a population of a specific species and confirm that it is the only one in a larger geographic area, currently and in the past, which would help identify previously sampled populations if sampling site descriptions are not accurate enough on their own.

2. Ancestral collections have to represent the genetic diversity

Franks and colleagues (2018) clarified that a representative and equal sampling of the genetic diversity of the target populations at both time points is crucial for valid comparisons in resurrection studies. This criterion can be ensured for the contemporary collection but the exact sampling protocol of the ancestors is often unknown when resourced from seed banks. However, some characteristics of the accessions may provide a sufficient proxy for valuing the genetic diversity within the ancestral collections. First, the former collectors are often known and can be consulted on their personal sampling protocols including the location and time of the year for sampling (pers. comm. Sandrine Godefroid). Second, scientists and collectors were already aware that ex-situ collections have to represent the genetic diversity of the populations and recommended rules for seed sampling (Brown 1989; Falk and Holsinger 1991; Guarino 1995; Way 2003), which were merged by the “European Native Seed Conservation network“ (ENSCONET) in 2009 (ENSCONET 2009). Thirdly, the number of collected individuals and/or the amount of seeds within stock are often included in the data sheets of the seed banks. For example, in the MSB the number of sampled plants is known for 71% of the accessions (Liu *et al.* 2018). Fourthly, the amount of seeds, depending on the species-specific seed set, can be used to estimate the number of sampled plants and high amounts may indicate that a larger number of individuals was sampled. Concerning this, 63% of the accessions of the MSB contain more than 1000 seeds (Liu *et al.* 2018) and Godefroid and colleagues (2011) found that 23-28% of the threatened species in 29 European seed banks are represented with more than 5000 seeds per accession. Fifthly, molecular data using ddRAD-SNP markers of the grown plants are helpful to decide whether seeds of the ancestors and descendants were collected in similar ways. Potential analyses are kinship comparisons within the temporal origins (Goudet *et al.* 2018) and comparisons of the allelic richness and private alleles (see Box 2, p. 46–48).

3. Suitable species – viable stored seeds, short lived species and sparse distribution

In order to revive plants from the stored seeds a high proportion has to be viable and germinable. If germination rates of the ancestral collections are low, there will be a high risk for “invisible fraction” due to the loss of existing genetic diversity within the stored seeds (Weis 2018). In general, “orthodox” seeds, which can be dried (15% equilibrium relative humidity) and stored at

low temperatures (-20°C), can survive for tens to hundreds of years (Walters *et al.* 2005; Liu *et al.* 2018; Solberg *et al.* 2020) and are therefore commonly stored in seed banks (Hay and Probert 2013). However, seed longevity differs a lot between species, families and geographical regions (Walters *et al.* 2005; Probert *et al.* 2009) and may depend on seed maturity at sampling (Hay and Probert 2013). Besides the characteristic that seeds should be "orthodox", the studied species should best be short lived or fast reproducing to increase the number of generations between the ancestors and their descendants, which would allow populations to rapidly adapt to environmental changes. Furthermore, species which have a locally sparse distribution are more suitable for resurrection studies using seed bank collections as intense gene-flow and crossing with neighbouring populations are likely reduced (Levin and Kerster 1975; Ellstrand 2014).

Considering these criteria, I am confident that seed bank collections can be used to turn the time consuming **"forward-in-time"** resurrection approach into a **"back-in-time"** approach to test for recent evolutionary changes in plant populations and to broaden the possibilities of climate change research (Fig.1). In contrast to classic resurrection studies, it is difficult in **"back-in-time"** approaches to achieve replication on species level by studying multiple populations. Such a multi-population design is necessary to make accurate predictions for single species. However, **"back-in-time"** resurrection studies may overcome this drawback by including multiple species, as the amount of available material from different species and taxa stored in seed banks is immense. In fact, multi-species studies are powerful to detect among-species patterns of responses to environmental changes and to address broad ecological questions (van Kleunen *et al.* 2014). In my work, I used the resources of seed bank stored and newly collected seeds from multiple, non-forest European plant species to test whether contemporary populations have recently evolved in response to increased drought frequencies and intensities within the last decades.

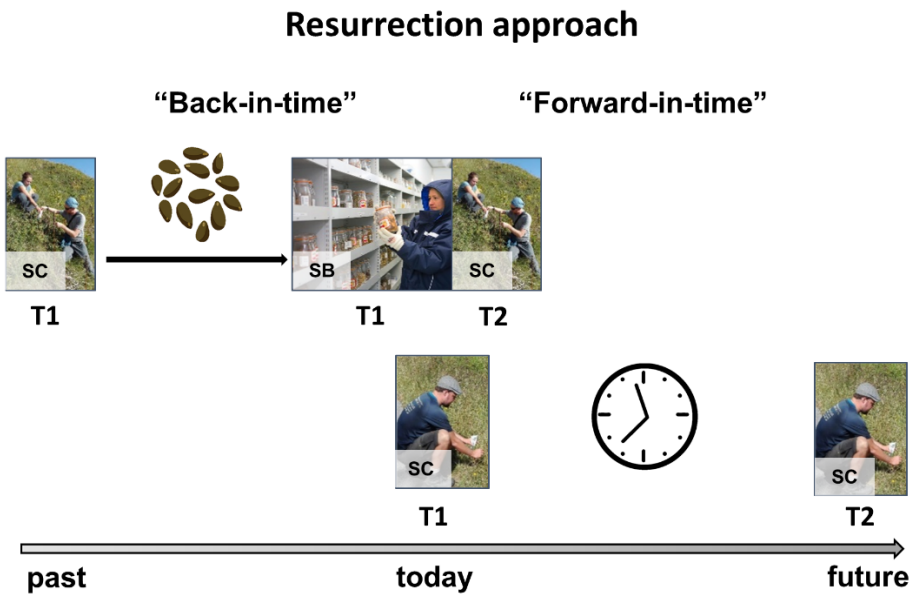

Figure 1 Graphic representation of the two presented different resurrection approach modes. T1 – ancestors, T2 – descendants, SC – seed collection, SB – seed bank (MSB, Royal Botanic Garden Kew)

Study system

Species selection

In 2017, I started collaborating with five seed banks from three different European biogeographic regions:

- Mediterranean: “Conservatoire Botanique National Méditerranéen de Porquerolles” (CBNMed, Hyères, France).
- European Alps: “Conservatoire. Botanique National Alpin de Gap-Charance” (CBN-Alpin, Gap, France)
- Temperate Europe: “Meise Botanic Garden” (MBG, Belgium), the “Botanical Garden of the University of Osnabrück” (BGO, Germany) and the “Berlin Botanic Garden and Botanical Museum” (BBG, Germany)

I initially chose 95 populations from 86 species from the catalogues fulfilling the above mentioned general criteria for using seed bank collections as a source for resurrection approach experiments. All the ancestral collections were sampled before 1998 and not more than 130 km away from the seed banks for resampling in spring and summer 2018. After several sampling

campaigns in 2018, I relocated 58 populations and had a sufficient amount of ancestral and contemporary seed material from 49 of those populations to conduct my experiments (Appendix Table A1, p. 127–129). After germinating ancestral and descendant seeds (Box 1, p. 21) I had to discard all accessions of the seeds originating from the European Alps as germination rates were rather small and seedling mortality was high. Therefore, I focused only on the two remaining biogeographic regions and used the species for which more than 30 seedlings per temporal origin have germinated for the following experiments (Table 1, Fig. 2)

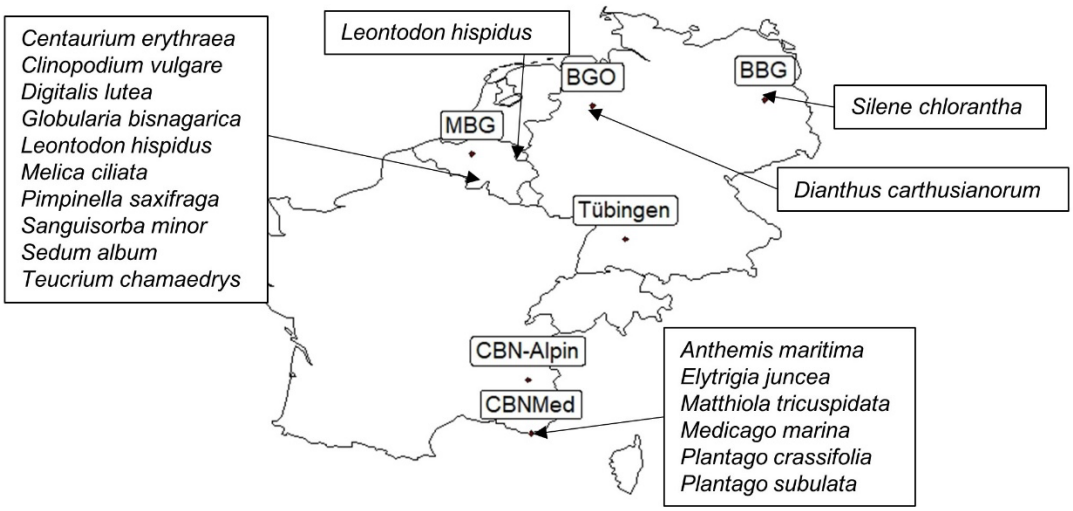

Figure 2 Location of the five seed banks and the experimental site at Tübingen (Germany) including the species' region of origin used within this thesis. CBNMed – "Conservatoire Botanique National Méditerranéen de Porquerolles", CBN-Alpin – "Conservatoire. Botanique National Alpin de Gap-Charance", MBG – "Meise Botanic Garden", BGO – "Botanical Garden of the University of Osnabrück", BBG – "Berlin Botanic Garden and Botanical Museum". Map was created with the R package *maps* (2018)

Climate change in the regions of the species' origins

The changes in climate mentioned earlier (p. 10) imply a general future increase in the frequency and duration of drought events in Southern and Central Europe (Sheffield and Wood 2007; Ruosteenoja *et al*. 2018; Samaniego *et al.* 2018; Spinoni *et al.* 2018). These models are important to predict future changes in plants, communities and species distributions (Prentice *et al.* 2007; Randin *et al.* 2009). The aim of my project was to investigate whether plants have already evolved in response to recent climate change. Data provided by the "Climatic Research Unit" (Camarillo-Naranjo *et al.* 2019; Harris *et al.* 2020) give more precise insights into climatic anomalies experienced by the populations between ancestral and contemporary seed collections. For the

Mediterranean species, average temperatures between March and July have increased by 1.1 °C and precipitation anomalies summed to a decrease of around 1.5 mm per year from 1985 until 2020 in comparison to 1900–1999. During the same period average temperatures in Belgium have increased by 0.9°C for the species originating from the region of Namur and by 1.1 °C for *Leontodon hispidus*. This increase by 1.1°C was also present close to Osnabrück and close to Berlin. In the Belgian regions and in Osnabrück precipitation in spring and summer has decreased by about 29 mm per year whereas Berlin experienced a decrease of 1.5 mm. These deficits in precipitation coupled with higher evapotranspiration due to warmer temperatures (Feng and Fu 2013) led to more frequent and severe droughts during the growing season in spring and summer within the last decades.

Table 1 Overview of all species which I investigated within this thesis including information about their family, their seed bank (CBNMed – "Conservatoire Botanique National Méditerranéen de Porquerolles", MBG – "Meise Botanic Garden", BGO – "Botanical Garden of the University of Osnabrück", BBG – "Berlin Botanic Garden and Botanical Museum", their region of origin (PACA – Provence-Alpes-Côte d'Azur (France), N. – Namur (Belgium), L. – Liège (Belgium), Ni. – Niedersachsen (Germany), B. = Berlin (Germany), their precise coordinates, their year when the ancestors were collected and the chapters where the species were included.

Species	Family	Seed bank	Locality (Region, Latitude, Longitude)	Year of collection	Chapters
Mediterranean					
Anthemis maritima L.	Asteraceae	CBNMed	PACA, 43.044977, 6.132747	1994	I, II
Elytrigia juncea (L.) Nevski	Poaceae	CBNMed	PACA, 43.044977, 6.132747	1994	I
*Matthiola tricuspidata (*L.) R. Br.	Brassicaceae	CBNMed	PACA, 43.044977, 6.132747	1994	I,II,III
Medicago marina L.	Fabaceae	CBNMed	PACA, 43.044977, 6.132747	1992	I,II
Plantago crassifolia Forssk.	Plantaginaceae	CBNMed	PACA, 43.044977, 6.132747	1994	I,III
Plantago subulata L.	Plantaginaceae	CBNMed	PACA, 43.026808, 6.148706	1997	I,II
Temperate					
Centaurium erythraea Rafn	Gentianaceae	MBG	N., 50.252214, 4.841663	1992	I,II
Clinopodium vulgare L.	Lamiaceae	MBG	N., 50.065255, 4.443902	1992	I,II,III
Dianthus carthusianorum L.	Caryophyllaceae	BGO	Ni, 52.208853, 7.954611	1993	I,II
Digitalis lutea L.	Plantaginaceae	MBG	N., 50.084677, 4.628027	1992	II
Globularia bisnagarica L.	Plantaginaceae	MBG	N., 50.305872, 4.893436	1992	I
Leontodon hispidus L.	Asteraceae	MBG	L., 50.792744, 5.672979	1995	I,II,III
Melica ciliata L.	Poaceae	MBG	N., 50.077245, 4.544916	1992	II
Pimpinella saxifraga L.	Apiaceae	MBG	N., 50.057297, 4.535364	1992	II
Sanguisorba minor Scop.	Rosaceae	MBG	N., 50.275593, 4.899947	1992	I
Sedum album L.	Crassulaceae	MBG	N., 50.275593, 4.899947	1992	II
Silene chlorantha (Willd.) Ehrh.	Caryophyllaceae	BBG	B., 52.598665, 13.224091	1980	I
Teucrium chamaedrys L.	Lamiaceae	MBG	N., 50.071527, 4.512406	1992	II

Objectives

Climate change challenges all ecosystems around the world and is a main driver for species evolution. Studying recent evolutionary changes in plant populations allows us to assess the rate of phenotypic evolution in rapidly changing environments and can give insight into potential trade-offs. This knowledge is crucial for evolutionary rescue, restoration and conservation. "**Forward-in-time"** resurrection approaches can be used to monitor such responses to climate change. However, such studies are time consuming and resource intensive. In order to overcome this limitation I adapted the **"forward-in-time"** approach using ancestral seeds stored in seed banks and freshly sampled seeds from the same populations, thus rendering this into a "**back-in-time"** approach. I aimed to test whether contemporary populations have recently evolved in response to increased drought frequencies and intensities within the last decades. I predicted to find general patterns of phenotypic change within different subsets of the 18 species from two European biogeographic regions. In order to study evolutionary shifts I conducted several common garden greenhouse experiments comparing ancestors with their descendants, in some of these using watering treatments to mimic drier conditions. During these experiments, I investigated plants in different life stages (seedlings, juvenile plants, reproducing plants). The experiments varied in their focus and complexity, ranging from multi-species experiments studying global patterns to seed family-based designs for more accurate, species-specific analyses.

The aim of **Chapter I** was to investigate shifts in phenology (flowering onset) and early growth using a multi-species design with 13 species. For two growing seasons from spring 2019 until summer 2020, I grew ancestors and descendants of seven temperate and six Mediterranean species under common garden greenhouse conditions. I measured their growth within the first three to four weeks, and scored their date of flowering onset. I hypothesised that the contemporary populations have evolved in parallel in order to escape from summer droughts by faster growth and advanced flowering onset. To test the impact of climate change, I included an aridity index in the analyses. Furthermore, I investigated whether the two different regions differed in their responses.

In **Chapter II** of my thesis, I focused on differences between ancestors and their descendants in early life stages, which are crucial for enduring population survival. During the seedling and juvenile stages, plants are especially susceptible to drought, and therefore supposedly under high selection pressure in drier environments. In a "seedling survival experiment" using seedlings of four Mediterranean species, I tested whether the descendants survived longer without watering

than their ancestors. I counted the days until a plant had died and correlated this observation with its size to detect relationships between survival and evapotranspiration. In a “watering response experiment”, I used juvenile plants of nine temperate species and subjected ancestors and descendants to well watered vs. dry conditions and compared their growth responses. I analysed both shifts in mean traits and in their plasticities. Finally, I discussed whether these responses might help to cope with increased drought frequencies or not.

In **Chapter III,** I studied species-specific differences between ancestors and descendants in four species (two from the Mediterranean and two from the temperate region): *Matthiola tricuspidata*, *Plantago crassifolia*, *Clinopodium vulgare* and *Leontodon hispidus*. In order to reduce potential storage and maternal effects I used seeds of refresher generations in this experiment. I established a full factorial design using a watering treatment (dry vs. well-watered conditions) combined with an herbivory treatment by injuring leaves. The aim of this study was to investigate whether the studied populations have evolved drought responses over the last two decades and how they may interact with co-occurring insect herbivory, which is an important stress for virtually all plant populations. Besides phenotypic analyses, including physiological measurements (leaf dry matter content and osmotic potential), I also tried to disentangle selective from random processes by performing comparisons of the quantitative genetic differentiation (Q_{ST}) with the neutral molecular differentiation (F_{ST}) (Merilä and Crnokrak 2001; McKay and Latta 2002).

Box 1

Resurrection via germination

Seeds of the ancestors were stored in the seed banks under appropriate conditions to safeguard viability. With the exception of *Plantago subulata*, which was ultra-desiccated and stored at 17°C, the Mediterranean species were dried after the collection and stored at 5°C at the CBNMed. Seeds of the temperate species and from the European Alps had been dried at 15% relative humidity and then stored at -20°C at Meise Botanic Garden, the Botanical Garden of the University of Osnabrück, Berlin Botanic Garden and Botanical Museum and at the CBN-Alpin. After recollecting the descendants in spring and summer 2018 and obtaining the ancestors in November 2018 the first step of my experimental work started in December 2018. In order to break potential seed dormancy (Baskin and Baskin 2004) all seeds were stratified in darkness at 5°C. For the Mediterranean accessions, I used 1% agar in 90 mm petri dishes to keep the seeds moist and had a stratification period of one week, as Mediterranean species often germinate in autumn without needing cold stratification (Milberg and Andersson 1998).
To break the physical seed dormancy of *Medicago marina*, I scarified the seeds of this species by softly scrubbing them with sandpaper (Royal Botanic Gardens Kew 2020). In order to reduce the growth of microbes during germination, I surface-sterilized the seeds for ten minutes with 3% sodium hypochlorite (NaOCl) and two drops of Tween20 per 200mL solution and washed them three times with sterilized water. After one week of stratification I germinated the seeds in a walk-in growth chamber (light intensity = 230 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 50% relative humidity) with a light/dark cycle of 8/16 hours and temperatures of 23/18 °C. In contrast to this procedure, I stratified the accessions from temperate Europe for two months and those from the European Alps for four months to simulate their natural climate conditions as spring germinating species. To reduce the growth of mould during this time I used seedling trays (TEKU®, TK1520 18.5 × 14 × 5.1 cm) filled with potting soil (Einheitserde®, BioLine, Pikiersubstrat) instead of petri dishes. From March to April 2019, I transferred the trays to the greenhouse and allowed the seeds to germinate at approx. 20 °C under a natural spring daylight regime. For each pair, ancestors and their descendants, I sowed a similar amount of seeds, which was at least 50 but also ranged up to 1000 (see **Chapter I** Table S1, p. 44–45). Using this approach combined with recording of the germination rates, I aimed to quantify and reduce possible invisible fractions during germination (Weis 2018).

Chapter I

Parallel evolution of advanced flowering across multiple European plant species in response to increased aridity over the last decades

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Abstract

Ongoing global warming and increased drought frequencies have a large impact on plant populations and potentially drive evolutionary adaptations. Historical comparisons, where plants grown from seeds collected in the past (“ancestors”) are compared to plants grown from freshly collected seeds from the same populations (“descendants”), are a powerful method to investigate such evolutionary changes across many taxa. When applied to multiple species simultaneously, historical comparisons can reveal recent parallel evolutionary shifts.

We used 21–38 year old seeds of 13 European plant species, stored in seed banks and originating from Mediterranean and temperate regions, for a greenhouse experiment that investigated shifts of flowering phenology, as a potential result of adaptive evolution to increased drought over the last decades. We additionally used single nucleotide polymorphism (SNP) markers to quantify relatedness and levels of genetic variation, and to characterize potential neutral processes and differences in sampling schemes. We found that, across species, descendants grew faster and advanced their flowering, and that these shifts were correlated with climatic changes at the population origins, suggesting that drought induced evolution of earlier flowering. In 6 out of the 13 species, however, the SNP markers detected strong differences in genetic variation and relatedness between ancestors and descendants, indicating that other evolutionary processes may have contributed to genetic changes. Our results suggest that climate change may have influenced the evolutionary trajectories of many plant species in different regions of Europe, and that flowering phenology may be one of the key traits that is rapidly evolving. Our study provides further evidence that seed bank collections, with some limitations, are a largely untapped resource for investigating the impact of global environmental changes on plant populations.

Introduction

Over the last decades climate change has increased dramatically in Europe (IPCC 2021). These changes include both higher temperatures (IPCC 2013) and shifts in precipitation patterns, which often imply increases in the frequency and duration of drought events, as is for instance the case in Southern and Central Europe (Ruosteenoja *et al.* 2018; Samaniego *et al.* 2018, Spinoni *et al.* 2018). Such drought events are often intensified by increased evapotranspiration induced by higher temperatures (Feng and Fu 2013). Plant populations will have to cope with such novel and more stressful environmental conditions (Anderson *et al.* 2012; Shaw and Etterson 2012; Fleta-Soriano and Munné-Bosch 2016) and are under an increased risk to go locally extinct (Thomas *et al.* 2004; Urban 2015).

To avoid local extinction, plant populations can migrate to track suitable conditions (Parmesan and Yohe 2003; Lenoir *et al.* 2008) or respond through phenotypic plasticity or adaptive evolution (Holt 1990; Hoffmann and Sgrò 2011). One example for plastic or evolutionary adjustments are shifts in phenological events such as leaf-out, flowering onset and time of fruiting, which are key events in plant species' life cycles and crucial for individual fitness. As phenology is frequently cued by environmental factors (Schwartz *et al.* 2006; Tang *et al.* 2016) it is likely that higher temperatures and more frequent droughts influence the timing of phenological events in plant populations. Due to their key role in many ecosystems, shifts in phenology may also impact pollinators, food webs and other ecosystem functions such as productivity or carbon cycling (Reilly *et al.* 1996; Chmielewski *et al.* 2004; Cleland *et al.* 2007; Tang *et al.* 2016).

Within the last two decades an increasing number of studies showed that plant populations are responding to climate change by shifting their phenology through phenotypic plasticity (Fitter and Fitter 2002; Primack *et al.* 2004; Panchen *et al.* 2012) or through adaptive evolution (Franks *et al.* 2007; Metz *et al.* 2020). Observational studies using field and/or herbaria data from multiple species illustrated that plant species in general advanced their flowering during the 19th and 20th century possibly related to increased temperatures. For example, Panchen and colleagues (2012) found that 28 species from the Greater Philadelphia region advanced their flowering by 2.7 days per 1°C rise in monthly minimum temperature within the last 170 years. Although such observational studies across multiple species are crucial to understand the relationship between climate change and shifts in phenology, it remains unclear whether the observed patterns are due to plastic responses or whether plant populations adapted evolutionarily to novel environmental conditions.

In order to investigate how climate change influenced recent evolution of plant populations' phenology, ancestral plants can be compared with descendant plants grown in a common garden,

using stored seeds and seeds sampled from the same population today (Orsini *et al.* 2013; Merilä and Hendry 2014; Franks *et al.* 2018). An increasing number of studies has used this “resurrection approach” to examine rapid evolution in response to increased temperature and drought. There are convincing examples showing that plants advanced their phenology towards an earlier flowering in order to avoid drought (Franks *et al.* 2007; Vigouroux *et al*. 2011; Nevo *et al.* 2012; Thomann *et al.* 2015). However, such studies are still rare because they require seed collections specifically designed for this purpose (Franks *et al.* 2018) and therefore often only cover single species. In addition, besides directional selection, genetic differentiation between ancestral and contemporary populations can also be influenced by other processes like bottlenecks, drift and/or immigration (Levin and Kerster 1975; Hay and Smith 2003; Ellstrand 2014; Hoban and Schlarbaum 2014). Molecular genetic data may help to sort these different factors out.

Although seed collections in seed banks have not been sampled for the purpose of conducting resurrection studies, they can be used in a similar way and thereby offer untapped resources for environmental change research on recent evolution (Everingham *et al.* 2021, Rauschkolb *et al.* 2022). As in seed banks numerous seeds are stored the same way for long time spans, they offer a suitable setup for multi-species studies to draw more generalised conclusions (van Kleunen *et al.* 2014), which may reveal parallel evolutionary responses to common drivers. To conduct experiments with seed bank material comparing ancestral populations with their descendants the amount of stored seeds should be high and information about their locality, their sampling protocol and their genetic diversity must be available (Rauschkolb *et al.* 2022).

In this study we adopted the resurrection approach in a multi-species experiment with seeds from seed banks. We compared 21 to 38 years old ancestors and their descendants from 13 different plant species, from Mediterranean and temperate regions of Europe. These populations experienced both increases in temperature and decreases in precipitation in spring and summer within the last 30 years. To investigate the evolution of phenology in these populations over the last decades we observed flowering onset and early growth traits of 15 plants per temporal origin (i.e. ancestors versus descendants) in a greenhouse experiment. To test the impact of climate change, we included the “De Martonne aridity index” (IDM) of the last six years before seeds of each population were collected in our analyses. In addition, we used genomic single nucleotide polymorphism (SNP) marker data following a ddRAD protocol to assess whether the genetic basis of ancestors and descendants was comparable. We hypothesised that descendants generally evolved an advanced flowering onset in comparison to their ancestors in order to escape from increasing temperatures and drought induced by climate change during the

last decades. We further hypothesised that plants which experienced drier climates within the last six years before seed collection showed stronger advances in flowering onset.

Methods

Seed collection

We obtained ancestral seeds from the seed bank at the Conservatoire Botanique National Méditerranéen de Porquerolles (CBNMed, Hyères, France), the Meise Botanic Garden (Belgium), the Botanical Garden of the University of Osnabrück (Germany) and the Berlin Botanic Garden and Botanical Museum (Germany). For the Mediterranean species (*Anthemis maritima*, *Elytrigia juncea*, *Matthiola tricuspidata, Medicago marina, Plantago crassifolia, Plantago subulata)* originating from the region of Hyères, Southern France, average temperatures between March and July have increased by 1.1 °C and precipitation anomalies in these months summed to a decrease of around 1.5 mm from 1985 until 2020 in comparison to 1900–1999. During the same period average temperatures in Belgium have increased by 0.9 °C for *Clinopodium vulgare*, *Centaurium erythraea, Globularia bisnagarica* and *Sanguisorba minor* (province Namur) and by 1.1 °C for *Leontodon hispidus* (province Liège) and also by 1.1°C close to Osnabrück (*Dianthus carthusianorum*) and close to Berlin (*Silene chlorantha*). In the Belgian regions and in Osnabrück precipitation in spring and summer has decreased by about 29 mm per year whereas Berlin experienced a decrease of 1.5 mm (data from the Climatic Research Unit; Camarillo-Naranjo *et al.* 2019; Harris *et al.* 2020).

We only used seed bank accessions which had detailed location records, had been stored for at least two decades and had a high number of stocked seeds. All ancestral seeds originated from visually well-demarcated populations that were sampled between 1980 and 1997 (Table S1). After bulking the seeds from different individuals, the seeds were cleaned and then stored under appropriate environmental conditions (see Box 1, p. 21) to preserve viability until we received the seed materials in November 2018. To obtain the descendants, we collected seeds of all species from the exact same populations in spring (Mediterranean species) and summer (temperate species) of 2018. We sampled, depending on the available number of fruiting plants, between 10 and 103 individuals (Table S1) per population and then bulked all seeds as ancestral seed collections were also mixed.

Germination and experimental design

Starting in December 2018, we germinated 100 seeds per temporal origin of the Mediterranean species and at least 50 seeds (exact number of seeds in Table S1) per temporal origin of the

temperate species (for more Information see Box 1, p. 21). After a sufficient number (>25) of seeds per temporal (i.e. ancestral vs. descendant) origin had germinated we transplanted 15 ancestral and 15 descendant seedlings into 9 × 9 cm pots (one plant per pot) with a 3:1 mixture of potting soil (Einheitserde®, BioLine, Topfsubstrat Öko torffrei) and sand (0–2 mm play sand, WECO GmbH) and placed them in the greenhouse. We always transplanted pairs of ancestor and descendant seedlings that were approximately of equal size to balance the start of the experiment. At this time, due to insufficient germination of six species, the number of experimental species from the Mediterranean region diminished from 12 sown to 9 transplanted and from the temperate region from 16 to 13 (Table S1). With the remaining 22 species, we performed a common garden experiment to compare phenotypic traits between ancestral and descendant populations.

During the whole experiment we blocked the pots by species but randomised pot positions every second week within the block. The greenhouse was set to a light/dark cycle of 12/12 hours and temperatures of 20/15 °C as upper/lower limits. Throughout the experiment, we watered the plants sufficiently and regularly (two to three times a week), always giving the same amount of water per species (100–200 mL, depending on the size of a species).

Three to four weeks after transplanting to pots, we measured on each plant one size trait (initial size: shoot length or rosette diameter), depending on the species' growth form. In April 2019 the first Mediterranean and in May 2019 the first temperate species started to flower. We recorded the day of the year (FT_{Ind}) of flowering onset per individual until the end of August. After the first year of our multi-species experiment seven out of 22 species had flowered. For these species, we finished the experiment and scored them as flowered in the first year (Table 1). To stimulate vernalisation we kept the 15 remaining species in a non-heated greenhouse from September to November 2019 and let temperatures drop to 5°C during December 2019 and January 2020. In February 2020, we cut off dead plant material and transferred the pots back to the experimental greenhouse, repeated the initial size measurements 3 weeks later and recorded the date of flowering per plant until July 2020. After running the experiment for about 1.5 years 13 out of 22 species – 6 from Mediterranean region and 7 from the temperate – had flowered (Table 1) and the data of these species were used in our analyses.

Table 1 Study species used in the experiment with details on the seed bank, year of collection, the 6-year-de Martonne aridity index (mm/°C), the year of flowering onset within our experiment and the number of individuals in the experiment and the flowering rate.

Species	Seed bank	Collection year	6- year - de Martonne ariditiy index (mm/°C)		Year of first flowering	Number of individuals and flowering rate	
			Ancestors	Descendants		Ancestors	Descendants
Mediterranean species							
Anthemis maritima	CBNMed	1992	25.3	35.3	second	15 (93%)	15 (87%)
Elytrgia juncea	CBNMed	1994	26.9	35.3	second	14 (64%)	17 (35%)
Matthiola tricuspidata	CBNMed	1994	26.9	35.3	first	20 (90%)	19 (100%)
Medicago marina	CBNMed	1992	25.3	35.3	second	13 (54%)	13 (62%)
Plantago crassifolia	CBNMed	1994	26.9	35.3	first	16 (81%)	16 (100%)
Plantago subulata	CBNMed	1997	32.2	35.3	first	13 (85%	14 (93%)
Temperate species							
Centaurium erythraea	Meise	1992	39.9	32.1	second	14 (57%)	14 (57%)
Clinopodium vulgare	Meise	1992	39.9	32.1	first	14 (100%)	15 (100%)
Dianthus carthusianorum	Osnabrück	1993	36.8	32.9	second	13 (46%)	11(82%)
Globularia bisnagarica	Meise	1992	39.9	32.1	second	12 (75%)	15 (80%)
Leontodon hispidus	Meise	1995	40.2	39.2	first	15 (100%)	15 (100%)
Sanguisorba minor	Meise	1992	39.9	32.1	second	8 (88%)	10 (80%)
Silene chlorantha	Berlin	1980	29.8	26.5	first	13 (69%)	13 (54%)

Statistical analyses

We standardised the flowering records per species by using the following formula:

$$FT_{St} = (FT_{Ind} + 1) - FT_{First}$$

where FT_{Ind} is the day of the year a plant flowered and FT_{First} is the day of the year the first plant of this species flowered. For the single-species analyses of flowering time, we used linear models which included temporal origin (ancestors vs. descendants) as explanatory variables and the initial size, depending on the year of flowering, as covariate. For the multi-species analysis of flowering time, we applied linear mixed-effects models using random slopes of species within the temporal origins as random factor, temporal origin, flowering year (flowered in the first year vs. flowered in the second year) and region (Mediterranean vs. temperate) as fixed factors and the initial size, which we standardised per species to a mean of 0 and a standard deviation of 1, as covariate. We included all interactions of the fixed factors as well as the initial size × flowering year interaction within our multi-species model. In order to improve model residuals we square-root-transformed the FT_{St} values.

To test the whether the temporal origins differed in their early growth, we ran linear single-species models with the species-specific initial size trait as response variable and the temporal origin as explanatory variables, and a multi-species linear mixed-effects model with the standardised size measurements as response variable and the same fixed and random factors as in the multi-species model for flowering time.

To investigate whether changes in climate during the last decades may have influenced evolutionary shifts in flowering onset in our tested populations we used interpolated monthly mean precipitation and temperature data from the locations of origin obtained from the Climatic Research Unit (Camarillo-Naranjo *et al.* 2019; Harris *et al.* 2020). With this data we also calculated the “De Martonne aridity index” (IDM, Pellicone *et al*. 2019) for the last six years before seeds of the population were collected (6-year-IDM), using the formula

$$6\ year\ IDM = \frac{P/6}{(T + 10)}$$

where P is the sum of precipitation within the 6 years (mm) and T (°C) is the mean annual temperature. Thus, lower values of the IDM indicate drier environmental conditions. Using this index is advantageous because it combines changes in temperatures and precipitation, which is important because these factors interact and should be considered together to understand the

impacts of climate change on shifts in phenology (Llorens and Peñuelas 2005; Bloor *et al.* 2010). This six-year period reduces the influence of environmental fluctuations within the long-term trends while simultaneously covering three to six generations of our experimental species because some of our species flowered within the first, other species in the second year in the greenhouse experiment. To test whether the aridity within the last 6 years before seed sampling was related to the mean and variation of flowering time, we ran linear models with the mean of the FT or the CV_{FT} per population as response variables, and IDM, temporal origin, flowering year, region and all interactions of the last three as fixed factors. To test whether the differences in aridity between the two temporal origins are related to the differences in flowering onset we calculated the IDM difference ($IDM_{Diff} = IDM_{Ancestor} - IDM_{Descendant}$) and the mean of FT and CV_{FT} difference ($FT_{Diff} = FT_{Ancestor} - FT_{Descendant}$, $CV_{Diff} = CV_{Ancestor} - CV_{Descendant}$) for each species. We ran linear models with the FT_{Diff} and the CV_{Diff} as response variables respectively and the IDM_{Diff}, the flowering year, the region and the interaction of the last two as explanatory variables. For all models, we visually checked the residuals for normality and heteroscedasticity. All analyses were done in R (Version 4.0.2) using the packages *plyr* for data management (Wickham 2011) and *lme4* (Bates *et al.* 2015) and *lmerTest* (Kuznetsova *et al.* 2017) for running our models using the *lmer()* function.

ddRAD-SNP analyses

In order to assess whether the genetic basis of ancestors and descendants was comparable, we quantified genetic relatedness and diversity of ancestral and descendant populations. We produced genomic single nucleotide polymorphism (SNP) marker data following a ddRAD protocol (Peterson *et al.* 2012, Appendix p. 125–126) and SNP genotyping with dDocent 2.6.0 (Puritz *et al.* 2014). The raw data have been deposited in the Europoean Nucleotide Archive under the accession number PRJEB47887 (https://www.ebi.ac.uk/ena/browser/view/PRJEB47887).

We assessed pairwise genomic relatedness among samples within ancestral and descendant populations using two estimators, genomic relatedness *G* (Yang *et al.* 2010) and the kinship estimator r^{β} (Goudet *et al.* 2018), which were applied to ancestral and descendant individuals in one population. In addition, we assessed genomic diversity within ancestral and descendant populations as allelic richness, Ar, thus correcting for differences in sample size (El Mousadik and Petit 1996) and through the number of private alleles (Kalinowski 2004).

Results

The germination rate across the studied species ranged from 25% to 98%. We observed low germination rates (≤ 30%) for both temporal origins of *A. maritima* and *P. crassifolia* and for the ancestors of *E. juncea*, *P. subulata* and *D. carthusianorum*. In general, seeds of descendants had higher germination rates, but large differences with their ancestors (>25 percentage points) were only present in three species (*M. marina*, *P. subulata* and *L. hispidus*, see Table S1).

Descendants grew faster within the first weeks of the experiment (initial size; $F_{1,13.7}$ = 4.5, p = 0.05) and this effect was even stronger in the species that flowered in the second year of the experiment (origin × flowering year; $F_{1,13.7}$ = 10.1, p = 0.006). In addition, we found a significant advance in flowering onset across 13 species, with descendants flowering on average 8.5 days earlier than the ancestors ($F_{1,13.8}$ = 5.6, p = 0.03). Furthermore, species which flowered in the second year showed a stronger advancing of flowering onset in comparison to the species which flowered in the first year ($F_{1,12.8}$ = 9.8, p = 0.008).

There were substantial differences among species in their magnitudes of evolutionary changes. Only two species showed significant differences between the temporal origins in the growth within the first weeks: *P. subulata* following and *A. maritima* not following the cross-species pattern (Table 2). Furthermore, descendants of five species flowered significantly earlier, whereas seven species showed no significant difference between the two temporal origins and only in *A. maritima* ancestors flowered earlier (Table 2). We also tested whether the initial size of the individuals influenced their flowering onset. Here, we found no correlation across species but we found significant results in four species, three of them (*M. tricuspidata*, *C. vulgare* and *S. chlorantha*) showing larger plants having more advancing in flowering onset and one species (*S. minor*) the opposite pattern (Table 2). In addition, an interaction between the initial size and the species‘ flowering year ($F_{1,276.7}$ = 9.3, p = 0.002) indicated that the initial size had a stronger positive effect on the flowering onset for the species which flowered in the first year.

Temporal origins which experienced drier environmental conditions (lower 6-year-IDM) within the last six years before the seeds were collected, flowered earlier (Fig. 1, F_1 = *7.4*, p = 0.01). There was no significant relationship between the CVs of flowering and the 6-year-IDM. We observed an increase in the aridity in the descendant populations, indicated by negative IDM_{Diff} in Fig. 2, which corresponded with advanced flowering onset within our experiment (Fig. 2A, F_1 = 10.4 p = 0.01). In addition, we found a trend of more drought-exposed descendant populations having higher CVs of flowering onset in comparison to their ancestors which were less drought-exposed (Fig. 2B, F_1 = *4.6*, p = 0.06).

Concerning the genomic relatedness, we found significant differences between ancestors

and descendants for genomic relatedness *G* in three out of 13 species (*P. subulata*, *G. bisnagarica* and *S. minor,* see Box 2, p. 46–48 Fig. 1A) and for the kinship estimator r^{β} in four species (*P. crassifolia*, *P. subulata*, *S. chlorantha* and *S. minor*, see Box 2, Fig.1B). In all of these cases, values were lower for the descendants. In addition, the differences of both estimators between the two temporal origins were conspicuously high but not significant for *M. marina* and *M. tricuspidata* (see Box 2, Fig. 1A,B). Furthermore, we found large differences in the allelic richness ($Ar_{des.}$ / $Ar_{anc.}$ > 1.1 or < 0.9) in most of the aforementioned species (*M. tricuspidata, M. marina, P. crassifolia*, *P. subulata, S. minor* and *S. chlorantha;* see Box 2, Fig. 1C), which was often accompanied by large differences in the number of private alleles and high F_{ST}-values (see Box 2, Table 1). For two species (*M. tricuspidata, M. marina*) $Ar_{des.}$ / $Ar_{anc.}$ was < 0.9 indicating higher diversity in the ancestors, whereas for the remaining four the descendants showed higher diversity.

Table 2 *F*- and *p*- values of the linear models for the single species analyses**.** Showing the effect of the temporal origin on the initial size as well as on the flowering onset, and the effect of the initial size on the flowering onset. The last column presents the shifts in flowering onset in days between the ancestors and the descendants.

Species	**Temporal origin (d.F. = 1)**				**Initial size on flowering onset (d.F. = 1)**		**Shift in flowering onset (days) (descendants - ancestors)**
	Initial size		**Flowering onset**				
	F	*p*	*F*	*p*	*F*	*p*	
A. maritima	**21.3**	**<0.001**	**5.1**	**0.03**	2.5	0.19	**8.3**
E. juncea	0.4	0.51	0.1	0.80	0.1	0.72	1.9
M. tricuspidata	3.3	0.08	2.2	0.15	**9.4**	**0.004**	-16.8
M. marina	0.0	0.85	0.3	0.57	0.2	0.64	-2.8
P. crassifolia	**23.5**	**<0.001**	1.0	0.32	0.4	0.52	1.7
P. subulata	0.4	0.53	**11.8**	**0.002**	1.3	0.27	**-22.9**
C. erythraea	0.4	0.55	**6.6**	**0.02**	1.7	0.21	**-23.4**
C. vulgare	3.8	0.06	**33.4**	**<0.001**	**31.3**	**<0.001**	**-15.8**
D. carthusianorum	0.0	0.92	0.1	0.74	0.7	0.41	-2.8
G. bisnagarica	0.0	0.86	**4.8**	**0.04**	0.1	0.79	**-6.2**
L. hispidus	1.2	0.29	1.0	0.33	0.1	0.77	3.6
S. minor	0.5	0.51	**32.9**	**<0.001**	**24.8**	**0.0003**	**-24.7**
S. chlorantha	1.1	0.31	3.1	0.10	**7.22**	**0.02**	-16.8

Figure 1 Standardised flowering onset of the 26 different accessions (13 species with ancestors and descendants) plotted against the De Martonne aridity index (6-year-IDM). The grey area depicts the 95% confidence interval of the linear regression.

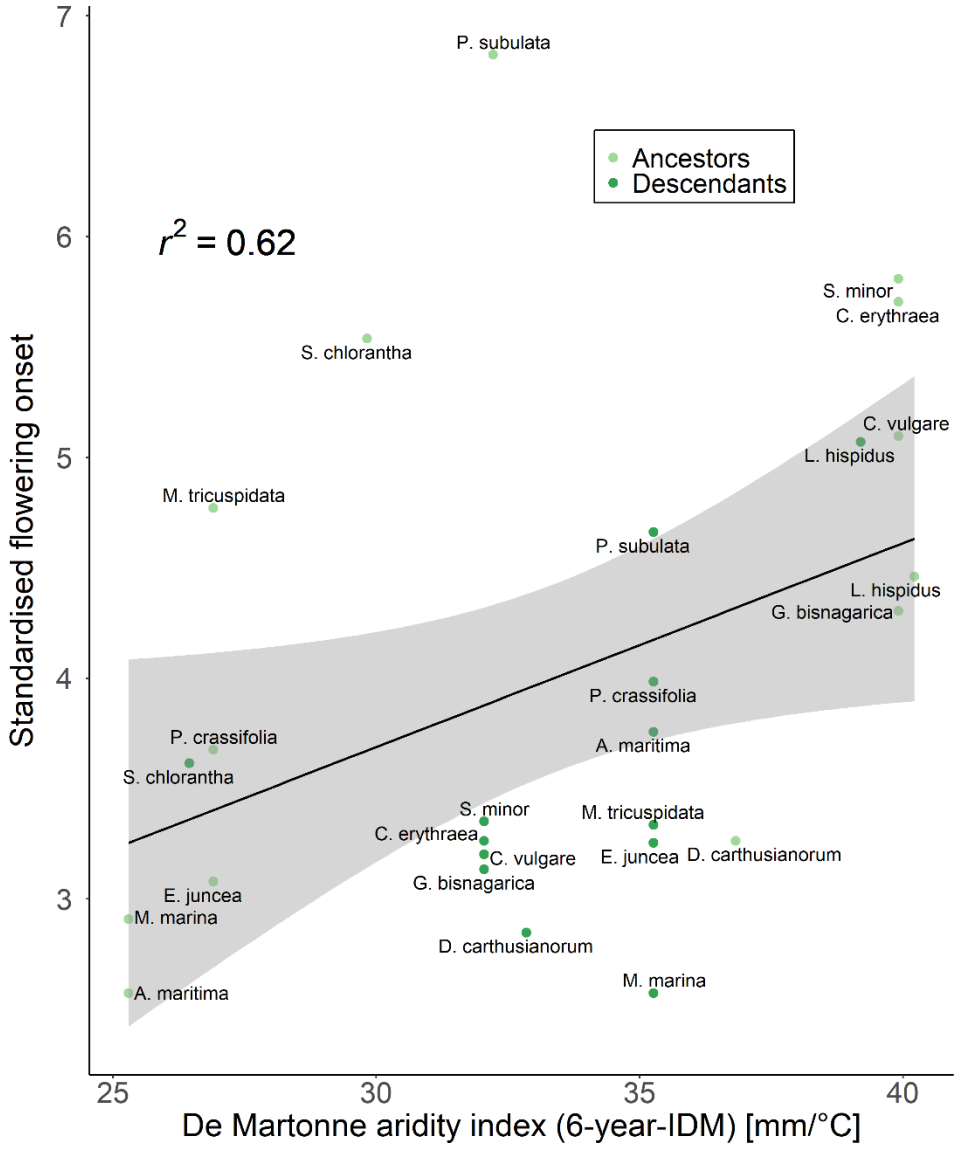
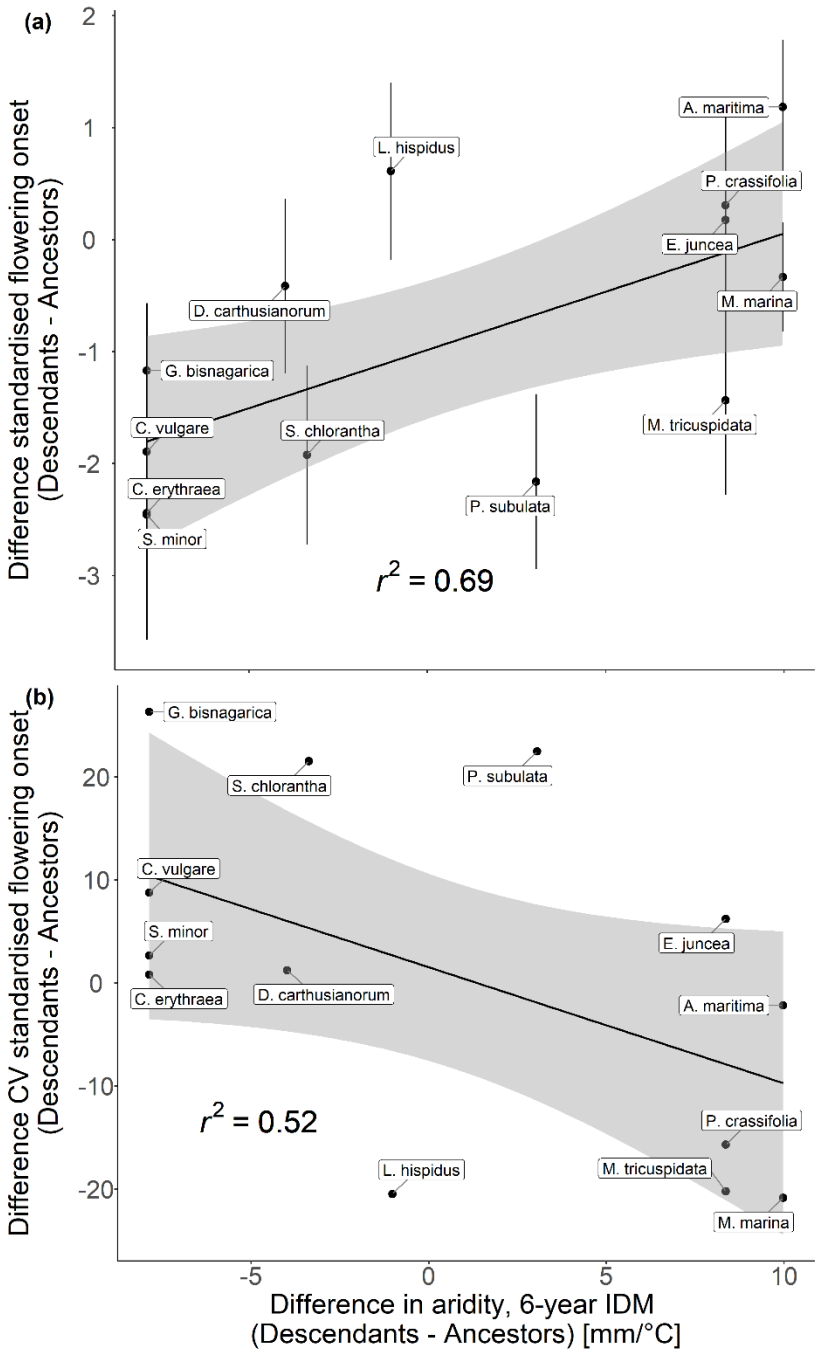


Figure 2 (A) Difference in the flowering onset of the two temporal origins and (B) the difference in the coefficient of variation (CV) as a function of the difference of aridity (6-year-IDM) between ancestors and descendants. Negative values therefore mean that the descendant climate is drier. The grey areas depict the 95% confidence intervals of the linear regressions and bars in (A) show the sum of the standard errors of both means.



The results of the molecular analyses (genomic relatedness *G,* kinship estimator r^{β}*,* allelic richness Ar and the number of private alleles) are presented in Box 2 (p. 46–48).

Discussion

Phenological differentiations between ancestral and contemporary plants

Our first aim was to investigate whether flowering onset in plants populations from Mediterranean and temperate origins has been evolutionary changed during the last decades. Indeed, we found that across the 13 studied species, descendants flowered earlier than their 21–38 years older ancestors, which was confirmed at the species level for five species and contradicted by only one species (*A. maritima*). This observation is in line with other studies which demonstrated that advanced flowering may reflect adaptation to an increasingly drier climate (Franks *et al.* 2007; Kigel *et al.* 2011; Metz *et al.* 2020) since early flowering individuals have a better chance to escape summer droughts. This relationship between drought and advanced flowering is further supported by our analyses of aridity index of sampling sites and flowering onset (Fig. 1), which revealed that temporal origins experiencing drier conditions prior to sampling also flowered earlier. In the Mediterranean species, the aridity index for the descendants (2012–2018) was always higher, which means less dry conditions, than that of the ancestors, which is at odds with the long term climatic pattern described in the introduction. Interestingly, the shift in flowering time was generally weaker for Mediterranean populations, but only once was the flowering shift in the opposite direction (*A. maritima*), as we would expect based on the change in aridity (Fig. 2A). This contrasts with the trend for temperate populations showing that descendants advanced their flowering (origin × region interaction for flowering onset; Fig. 2A). This observation is to some degree in line with other studies showing that plants in Mediterranean regions flower in general earlier but the shifts during the last decades in response to global warming were smaller compared to plants in temperate regions (Schwartz *et al.* 2006; Templ *et al.* 2017). We also found a trend that temporal origins with lower IDM had higher CVs of flowering onset in comparison to their ancestors or descendants with less dry IDM (Fig. 2B). Increased drought could have formed greater environmental heterogeneity including local microclimatic patterns (Altvater *et al.* 2011). Here, the CV of flowering estimates the genetically based phenotypic variability, which is generally expected to be higher under more variable environmental conditions (Karbstein *et al.* 2019; 2020).

For some species (*P. subulata*, *C. erythraea* and *S. minor*) the advances in the flowering onset for the descendants, measured in the common garden, were very large (>20 days) with a rate of shifting of about 1 day per year which is remarkably high in comparison to other studies. For example, Metz and colleagues compared populations of *Biscutella didyma* which experienced artificial watering regimes for 10 years and found earlier flowering of 3–4 days (rate: 0.3–0.4 days per year) in the drought-treated population in comparison to the control population when grown

in a common garden (Metz *et al.* 2020). These ranges of flowering shifts were also confirmed in other studies with *Hordeum spontaneum* showing a shift of 0.39 days per year (Nevo *et al.* 2012) or with *Centaurea cyanus* shifting 0.17 days per year (Thoman *et al.* 2015). However, Franks and colleagues detected shifts of 1.8 and 8.6 days comparing ancestors of *Brassica rapa* collected in 1997 with their descendants collected in 2004 after several years of drought indicating rates of 0.27 and 1.22 days per year (Franks *et al.* 2007) which would be in line with our results for *P. subulata*, *C. erythraea* and *S. minor*.

Cross species analyses showed, that the descendants grew faster within the first three weeks of the experiment compared to their ancestors, suggesting that the descendants have accelerated their life cycle not only through advanced flowering but also through faster growth. Herbaceous plants have to reach size thresholds before they start flowering (Mooney *et al.* 1986; Sun and Frelich 2011) and therefore fast-growing plants can flower earlier. However, in our experiment plants of only three species (*M. tricuspidata*, *C. vulgare*, *S. chlorantha*) with higher initial size also flowered earlier and there was no cross-species effect. Interestingly all three species flowered in the first year of the experiment and we also found an initial size × flowering year interaction showing that the flowering onset of first years' species is more strongly influenced by the initial size. We therefore argue that the connection between fast growth and early flowering is more important for the species which flowered in the first year of our experiment as these are comparable to ruderal or annual species which rapidly fulfill their life cycle by fast growth and early flowering (Grime 1977; Fitter and Fitter 2002). In contrast, the species which flowered in the second year are less influenced by the initial size. As those species perceive a period of stasis over winter, their flowering onset might be synchronized by the start of the growing period after winter reducing the importance of the initial size. To investigate whether the increased growth of the descendants within the first three weeks supported the advanced flowering, we should have measured other traits like size of the plants at flowering or portion of investments into vegetative and reproductive biomass. We also found that the species which flowered in the second year did so within a shorter time after cutting dead plant material after the winter and moving the pots to the warm greenhouse compared to the time between transplanting and flowering of species which flowered in the first year. This may simply be due to species differences, due to the fact that these species could draw on stored metabolites in spring, or due to any environmental differences in the greenhouse between the two years.

Differences in the genetic basis of ancestors and descendants

The fundamental question with our approach is the genealogical relationship between the newly collected seeds, i.e. descendants and those collected >20 years ago, i.e. “ancestors”. Apart from selective processes, neutral processes can affect genetic variation when comparing “ancestral”, stored seeds and freshly collected “descendant” seeds with particular effects on relatedness, genetic variation and private alleles. Molecular genetic markers can help to elucidate this question. Population bottlenecks occurring after the seed collection of descendants could have led to genetic drift, thus strongly changing the genetic makeup, in particular reducing genetic variation and increasing relatedness (Hay and Smith 2003; Hoban and Schlarbaum 2014). Similarly, gene flow could have led to immigration of new alleles from adjacent populations, thus increasing genetic variation and reducing relatedness (Levin and Kerster 1975; Ellstrand 2014). In the extreme case a population collected 20 years ago could have gone extinct in between and could have been recolonized from some other source, thus erasing the recent ancestor-descendant relationship. On the other hand, temporal variation and seed bank dynamics could have affected the populations during the years of seed collection leading to genetic differences. It can also be hypothesised that in self-compatible species the degree of outcrossing and thus the relatedness of seeds may vary among years, e.g. depending on pollinator activity. All these processes, although affecting the genetic pattern, still only involve natural processes within and among populations. In addition, the sampling scheme affects the genetic variation encompassed in a seed collection, in particular the number of mother plants seeds were collected from, their level of inbreeding and the equality of relative contributions of each mother plant in the bulked seed (Hay and Smith 2003; Hoban and Schlarbaum 2014).

In six species we found high differentiation between ancestors and descendants and strong differences in the number of private alleles. A loss of alleles accompanied by an increase of relatedness was found in *M. marina* and *M. tricuspidata*. As the seed collection of the descendants definitely included a large number of mother plants it may be hypothesised that population bottlenecks and disturbance have occurred between the two sampling dates in these populations. A gain of alleles and genetic variation, accompanied by reduction of relatedness was found in *P. subulata*, *S. minor*, *S. chloroantha*, *P. crassifolia*. In these species, gene flow, immigration, metapopulation processes, or different sampling schemes seem were likely involved and have led to strong genome wide genetic differentiation between the two generations. E.g. we could expect that less or more closely related plants of the ancestors were collected in the past (Hay and Smith 2003; Hoban and Schlarbaum 2014). With regard to this, we cannot rule out invisible fractions (Weis 2018) for *P. subulata,* as the germination rate of the ancestors was clearly

smaller (29%) in comparison to the descendants (78%). Thus, it is quite likely that the investigated individuals only represent a subset of the existing phenotypes. Alternatively, demographic events like gene flow from nearby populations may be the reason for the increased diversity in the descendants (Levin and Kerster 1975; Ellstrand 2014). Summarising for these six species, as both neutral processes and selection by climate change were active, the observed phenotypic change cannot unequivocally be attributed to adaptive evolution. Thus, it remains unclear whether the observed differences in flowering onset for these species are due to selective processes to escape from drought stress in summer or due to differences in the genetic basis of the two different time points.

In the rest of the species, i.e. *A. maritima*, *E. juncea*, *C. erythraea*, *C. vulgare*, *D. carthusianorum*, *G. bisnagarica*, *L. hispidus*, both ancestral and descendant populations showed low F_{ST} values, a similar level of genetic variation and relatedness (with uncertainties for *G. bisnagarica*) suggesting that in these species neutral processes are unlikely to have affected the populations. We thus assume that in those the descendants are related in direct line with ancestors leaving selection as the main and dominant driver of evolution. For these species it is therefore likely that the differences in flowering onset between ancestors and descendants are the result of directional selection responding to changes in climate (Franks *et al.* 2007; Kigel *et al.* 2011; Metz *et al.* 2020).

Using seed bank material for historical comparisons

As we used a common garden approach with controlled conditions for both temporal origins we are confident that the observed differences between ancestors and descendants are due to potentially adaptive evolutionary processes and not due to plastic responses. Since flowering phenology is often highly responsive to environmental cues or threshold values, flowering time differences from observational studies may to a large extent reflect plastic responses (Fitter and Fitter 2002; Primack *et al.* 2004; Panchen *et al.* 2012). Our historical comparison overcomes this problem but also has some weaknesses. First of all, the material from the seed banks was not collected with the aim to conduct resurrection approach experiments. Therefore, the sampling design and effort may have been different between ancestors and descendants and invisible fractions could appear during storage. Also, ancestral seeds were bulked, which prompted us to do the same with descendant seeds sampled in 2018. Thus, in four species (*P. subulata*, *S. minor*, *S. chlorantha*, *P. crassifolia*) the relatedness strongly decreased and in two species (M. *tricuspidata*, *M. marina*) increased over time likely indicating different sampling schemes or bottlenecks. For the other species we are confident that the seeds of the remaining studied

species could be used in our historical comparison in a way similar to resurrection studies. Reasons for that are (1) the aim of the collectors to maximize the number of sampled individuals already in the past, (2) the large number of seeds within the stored lots and (3) the similar relatedness of ancestors to that of descendants for these species, which was shown by molecular marker results. The latter result provides further support for similar sampling procedures in the past compared to today and indicates that a sufficient number of seeds was sampled during both periods (Rauschkolb *et al.* 2022).

In contrast to standard practice in resurrection studies, we did not grow a 'refresher generation' of ancestors and descendants before we conducted our main experiment (Franks *et al.* 2018). We therefore cannot exclude potential maternal effects, which are ubiquitous among plants (Roach and Wulff 1987; Mousseau and Fox 1998). It is known that offspring phenotypes can be influenced by the environmental conditions of the maternal lines (Sultan 1996; Galloway 2001). However, since mother plants mainly have strong influence on seed characteristics, such as seed provisioning, maternal effects may play an especially important role during germination (e.g. germination time) and seedling establishment (e.g. survival and developmental rate) (Roach and Wulff 1987; Galloway 2001; Gimeno *et al.* 2009). Differences in germination and seedling establishment traits may, still, have trickle-down effects on later life-stages and traits (e.g. competitive ability, flowering time) by shifting the offspring's life-cycle (Galloway 2002). However, several studies that included plant origins with strong environmental differences and that compared offspring from a refresher generation with parents from naturally collected seed found no or only minor differences in plant growth traits between the generations (Hodgin and Rieseberg 2011; Teller *et al.* 2014; Metz *et al.* 2015; Everingham *et al.* 2021) and that maternal effects diminish rapidly with plant development (Bischoff and Müller-Schärer 2010). Besides growing a refresher generation, maternal effects can also to some extent be accounted for by including initial seed or seedling size measurements in statistical analyses (Latzel 2015). Although we did not grow a refresher generation to reduce potential maternal effects, we always transplanted pairs of ancestor and descendant seedlings that were approximately of equal size, which is an alternative to accounting for seed or seedling size in statistical analyses. Thus, we are confident that our experimental design is valid, because even if maternal effects are occurring in our experiment, their contribution to the observed differences in flowering onset, a trait occurring relatively late in plant development, are likely small. Moreover, if maternal effects would be stronger than evolutionary changes in flowering time, a general pattern of advanced flowering across all populations cannot be expected given the random selection of species with different localities (i.e. soils and microclimates) and – for the descendants – different sampling years and time of the

year. Despite the possibility of maternal effects in our study, our decision to skip a refresher generation was made for practical reasons, since growing a refresher generation would have taken up to three years given that a portion of the species only started flowering in the second year. Only in this way could we include 13 species in this study and draw cross-species conclusions on parallel evolution of flowering time.

Conclusions

It is predicted that ongoing climate change influences the evolution of plant populations. So far experiments testing this have focused mostly on single species and studies investigating a large set of species are rare. Such a multi-species approach is valuable because it can demonstrate parallel evolutionary responses to broad-scale environmental changes and can reveal different evolutionary trajectories due to different selection pressures in different regions. In this study, we adjusted the “resurrection approach” and used historical comparisons comparing descendants and their ancestors of 13 different species from two different biogeographic regions in Europe.

We observed that descendants accelerated their reproductive cycle by faster growth and advanced flowering and that flowering partly correlated with the short-term climatic backgrounds of the temporal origins. Although we consider for some species that the observed phenotypic differences between ancestors and descendants may involve differences in the genetic basis of the two different time points, we still found consistent results across species, the temporal origins and their climatic developments. Therefore, it would be unlikely that part or all observed patterns in plant responses were due to neutral evolutionary processes (i.e, drift and gene flow) or unintentional selection during seed collection campaigns and experimental set up. Thus, we conclude that selection has driven most of these evolutionary changes. Our results suggest that climate change may have already influenced the evolutionary trajectory of many plant species in different regions of Europe. With our study we also demonstrated that historical comparisons using seed bank material are a powerful tool for studying rapid evolution in multiple plant species. We recommend that, if possible, future studies should use seeds from refresher generations to minimize possible maternal or storage effects. Furthermore, fitness measures should be incorporated to disentangle adaptive from non-adaptive and maladaptive responses to recent climate change.

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Supplementary material

Table S1 Sampled study species with details on the year of collection, number of sampled individuals in 2018, the number of seeds used and the germination rates

Species	Collection year	Number of sampled individuals in 2018	Number of used seeds and germination rate	
			Ancestors	Descendants
Ammophila arenaria	1994	16	100 (20%)	100 (20%)
Anthemis maritima	1992	80	100 (25%)	100 (30%)
Anthyllis barba-jovis	1992	19	100 (60%)	100 (75%)
Elytrigia juncea	1994	25	100 (25%)	100 (45%)
Euphorbia peplis	1998	20	100 (0%)	100 (0%)
Matthiola tricuspidata	1994	15	100 (95%)	100 (98%)
Medicago marina	1992	59	100 (45%)	100 (90%)
Palensis maritima	1994	10	100 (28%)	100 (29%)
Plantago crassifolia	1994	10	100 (28%)	100 (29%)
Plantago subulata	1997	103	100 (29%)	100 (78%)
Pseudorlaya pumila	1992	26	100 (0%)	100 (0%)
Silene nicacensis	1980	19	100 (1%)	100 (3%)
Centaurium erythraea	1992	20	200 (52%)	1000 (49%)
Clinopodium vulgare	1992	47	200 (75%)	200 (97%)
Dianthus carthusianorum	1993	20	100 (26%)	200 (48%)
Digitalis lutea	1995	20	500 (20%)	500 (30%)
Digitalis purpurea	1990	20	200 (100%)	500 (20%)
Globularia bisnagarica	1992	12	100 (49%)	50 (54%)
Hypericum montanum	1997	20	250 (20%)	100 (100%)
Leontodon hispidus	1995	20	300 (32%)	300 (75%)
Lithospermum officinale	1996	20	150 (2%)	150 (2%)
Melica ciliata	1992	21	200 (75%)	150 (50%)
Pimpinella saxifraga	1992	20	200 (50%)	200 (25%)
Rhinanthus minor	1990	20	200 (1%)	200 (0.5%)

Table S1 continued

Species	Collection year	Number of sampled individuals in 2018	Number of used seeds and germination rate	
			Ancestors	Descendants
Sanguisorba minor	1992	20	100 (53%)	50 (60%)
Sedum album	1992	20	500 (20%)	500 (20%)
Silene chlorantha	1980	25	150 (32%)	500 (47%)
Teucrium chamaedrys	1992	20	200 (20%)	300 (20%)

Box 2

Comparing genomic relatedness and diversity between ancestral and descendant populations

Molecular analyses of the ancestral and descendant populations using SNP data obtained from leaf samples followed by a ddRAD protocol (Peterson *et al.* 2012, Appendix p. 125–126) can be useful to qualify potential evolutionary processes within the two temporal origins. We assessed pairwise genomic relatedness among samples within ancestral and descendant populations using two estimators, genomic relatedness *G* (Yang *et al.* 2010) and the kinship estimator r^{β} (Goudet *et al.* 2018), which were applied to ancestral and descendant individuals in one population. Both estimators are relative measures of relatedness based on genomic marker data, which take a value of zero for pairs of randomly related individuals, positive values for more closely and negative values for less closely related than expected at random given allele frequencies of the population. For each species, we tested for significant differences of pairwise relatedness between ancestral and descendant populations using analysis of variance. In addition we assessed genomic diversity within ancestral and descendant populations as allelic richness, thus correcting for differences in sample size (Ar, El Mousadik and Petit 1996), and through the amount of private alleles (Kalinowski 2004).

Concerning the genomic relatedness, we found significant differences between ancestors and descendants for three species (*Plantago subulata*, *Globularia bisnagarica* and *Sanguisorba minor*, Fig. 1A) and for r^{β} in four species (*Plantago crassifolia*, *Plantago subulata*, *Silene chlorantha* and *Sanguisorba minor*, Fig. 1B). In addition, the differences of both estimators between the two temporal origins were conspicuously high but not significant for *Medicago marina* and for *Matthiola tricuspidata* (Fig. 1A,B). Furthermore, we found large differences in the allelic richness ($Ar_{des.}/AR_{anc.} > 1.1$ or < 0.9) in *M. tricuspidata*, *M. marina*, *P. crassifolia*, *P. subulata*, *S. minor* and *S. chlorantha* (Fig. 1C), which was often accompanied by large differences in the number of private alleles and F_{ST}-values (Table 1). For two species (*M. tricuspidata, M. marina*) $Ar_{des.}/AR_{anc.}$ was < 0.9 indicating higher diversity in the ancestors, whereas for the remaining four the descendants showed higher diversity. Considering these observations, we conclude that the two temporal origins of *M. marina*, *P. crassifolia*, *P.* subulata, *S. minor* and *S. chlorantha* possibly do not represent a comparable genetic basis for running reliable experiments. On the one hand we could expect for *P. crassifolia*, *P. subulata*, *S. minor* and *S. chlorantha* where allelic richness was larger in the descendants, that less plants of the ancestors were collected or selection took place during storage in the seed banks.

Alternatively, gene flow from nearby populations may be the reason for the increased diversity. On the other hand, descendants of *M. marina* showed lower diversity, which might be the result of bottleneck events within the last years or really large and broad-range sampling of the ancestors.

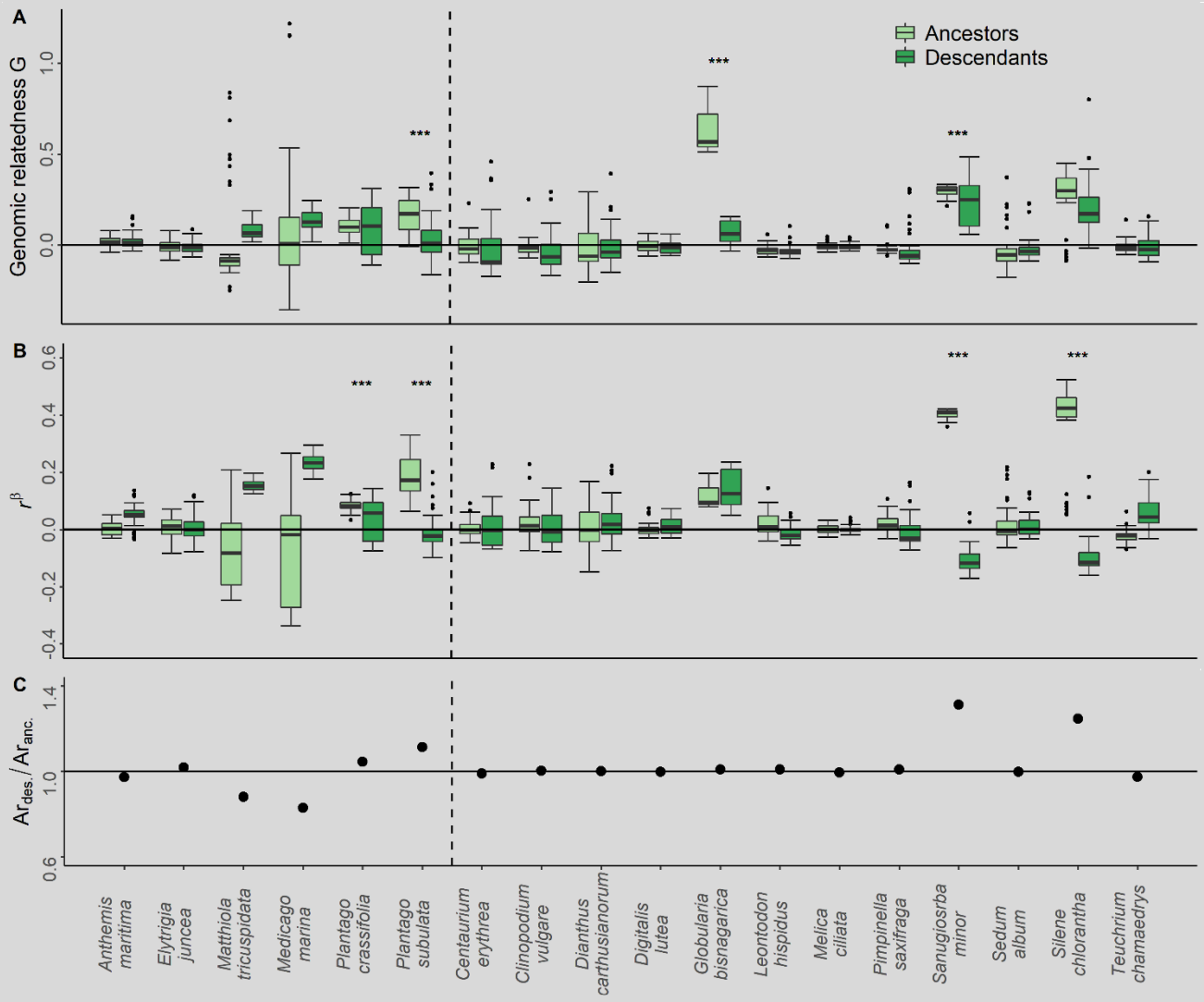

Figure 1 Comparative genomic SNP marker analysis for ancestral / descendant population pairs of 18 plant species: six species from the Mediterranean and twelve from the continental European region. A: genomic relatedness *G*; B: kinship coefficient r^{β}; C: relative genomic diversity of descendant populations ($Ar_{des.}$ /$AR_{anc.}$)

Table 1: Overview of molecular data results of all species which I investigated within this thesis including information about the number of found loci, the F_{ST} and the differences in the number of private alleles

Species	Chapters	Number of loci	F_{ST}	Delta number of private alleles (descendants – ancestors)
Mediterranean				
Anthemis maritima	I, II	3480	0.065	- 156
Elytrigia juncea	I	204	0.014	6
Matthiola tricuspidata	I,II,III	2180	0.087	- 533
Medicago marina	I,II	2693	0.156	- 1038
Plantago crassifolia	I,III	5785	0.148	673
Plantago subulata	I,II	4976	0.157	1052
Temperate				
Centaurium erythraea	I,II	3018	0.044	- 77
Clinopodium vulgare	I,II,III	3179	0.043	1
Dianthus carthusianorum	I,II	3021	0.056	- 29
Digitalis lutea	II	2039	0.016	1
Globularia bisnagarica	I	528	0.277	2
Leontodon hispidus	I,II,III	4114	0.005	120
Melica ciliata	II	1316	0.004	- 12
Pimpinella saxifraga	II	4813	0.015	47
Sanguisorba minor	I	6028	0.319	3003
Sedum album	II	3987	0.038	3
Silene chlorantha	I	1971	0.255	633
Teucrium chamaedrys	II	5259	0.038	- 271

Summing up the molecular analyses of the SNP data comparing ancestors and descendants of the 18 studied species we found no evidence for large differences in the genetic basis of the two temporal origins in eleven, slight differences in one (*G. bisnagarica*) and striking differences in six species (*M. marina*, *M. tricuspidata*, P. *crassifolia*, *P. subulata*, *S. chlorantha*, S*. minor)*. We conclude that molecular methods, in addition to proper collection of information, can be a useful tool to evaluate the potential of seed bank collections for experimental set-ups adapting the resurrection approach. However, as we only compared two different time points of single populations it is difficult to draw distinct conclusions. In order to interpret the differences between ancestors and descendants more properly, spatial (populations close to the collected population) and temporal patterns (more time points) have to be considered.

Chapter II

Historical comparisons show evolutionary changes in drought responses in European plant species after two decades of climate change

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Abstract

Plants must continuously respond to environmental changes, and a timely question is whether and how populations respond to ongoing global warming and increased drought frequencies and intensities. Plants can respond either through migration or through phenotypic plasticity or their populations can adapt evolutionarily, which encompasses the evolution of trait means and of trait plasticity. One way to detect such evolutionary changes within plant populations is through historical comparisons where plants grown from seeds collected in the past ("ancestors") are compared to freshly collected seeds from the same populations ("descendants") in common garden experiments. We used 21–26 year old seeds stored in seed banks for two multi-species experiments that investigated changes in phenotypic traits and their plasticity conferring drought tolerance in early life stages of European plant species. In the first experiment, we used seedlings of four Mediterranean species, ceased watering and recorded their day of mortality. In the second experiment, we studied phenotypic responses to drought in juvenile plants of nine species originating from temperate regions in Europe. In one of four species in the first experiment, descendants survived significantly longer without watering and were smaller than their ancestors. In the second experiment, descendant plants were generally taller under well-watered conditions but smaller under drought than their ancestors, thus showing stronger plasticity. Our historical comparisons suggest that some populations have likely evolved through changes in trait means and plasticity in ways consistent with adaptation to increased drought. Using seed bank material for historical comparisons has several weaknesses, such as unknown sampling protocols or invisible fractions. However, we show how accurately sampled and stored seed bank collections can be used similar to the resurrection approach for investigating rapid evolutionary processes in early life stages of plants under climate change.

Introduction

Climate change has increased dramatically over the last several decades (IPCC 2021), and plant populations are already responding (Peñuelas and Filella 2001; Parmesan and Yohe 2003). Projections for Europe forecast that, during the 21st century, annual precipitation sums will further increase in the north and decrease in the south (IPCC 2013). For central and western Europe, precipitation is expected to increase in the winter and decrease in the summer (IPCC 2013), leading to more droughts in the growing season. Moreover, the higher temperatures will lead to higher evapotranspiration (Feng and Fu 2013). These changes in environmental conditions will likely increase the frequency, duration and geographic extent of drought events in Southern and Central Europe (Ruosteenoja *et al.* 2018; Samaniego *et al.* 2018; Spinoni *et al.* 2018).

Changes in water availability and more frequent droughts are strong stressors for plants (Jaleel *et al.* 2009; Fleta-Soriano and Munné-Bosch 2016), and many plant populations may not be adapted to these novel conditions (Anderson *et al.* 2012; Shaw and Etterson 2012). To avoid extinction, some plant populations may migrate to track suitable conditions whereas others may respond through phenotypic plasticity or adaptive evolution (Holt 1990; Hoffmann and Sgrò 2011). Such evolutionary adjustments could be the result of selection of better adapted phenotypes and might involve reduced growth, reduced evapotranspiration (Kusaka *et al.* 2005; Borrell *et al.* 2014), or increased root-shoot ratio (Sharp and LeNoble 2002; Aroca 2012) to promote water uptake. In environments that become generally drier, constitutive changes in such traits may be adaptive. However, drought events are often periodic, which would render the ability to change functional trait values through phenotypic plasticity a better strategy than to evolve constitutive changes in mean traits (Sultan and Spencer 2002; Alpert and Simms 2002; Gianoli and Valladares 2012), especially in environments with strong climatic variability (Scheepens *et al.* 2018). Still, studies on the effects of climate change on plant populations often only consider changes in mean climate conditions (Bertrand *et al.* 2011), despite the strong evidence for increased climatic variability both among and within years (IPCC 2013; Gherardi and Sala 2019), specifically more heavy rain events followed by longer dry periods in many regions (Kharin *et al.* 2007).

Phenotypic plasticity itself can also evolve and is thought to be selected for particularly in spatially or temporally variable environments (Ackerly *et al.* 2000; Richards *et al.* 2006). For example, Lázaro-Nogal *et al.* (2015) showed in a common garden study with *Senna candolleana* that populations from environments with stronger interannual precipitation variation had a higher plasticity in growth traits. A similar observation was made by Gianoli and Gonzáles-Teuber (2005) who showed that plasticity in leaf area, leaf shape, leaf area ratio, and foliar trichome density in

Convolvulus chilensis was highest for plants from the population with the highest interannual variation in precipitation. Thus, increased climatic variability appears to be associated with systematic, and presumably adaptive, changes in phenotypic plasticity of plants. The fate of plant populations will thus depend on their ability to adapt to altered climatic variability and increased drought intensities through evolution of plasticity and/or constitutive adaptation to drought.

A powerful method to test for recent evolution – whether in trait means or in their plasticity – is to compare ancestors with their descendants by using stored propagules such as seeds (Franks *et al.* 2007; Orsini *et al.* 2013; Merilä and Hendry 2014; Franks *et al.* 2018). If ancestors can be revived, the resulting plants can be compared to contemporary individuals raised from propagules sampled from the same population. Growing ancestors and descendants together under common conditions then allows for direct tests for heritable trait differentiation among temporally separated populations (Franks *et al.* 2007, 2008). Understanding how populations and species responded evolutionarily in the past is extremely valuable for making predictions for future population and species responses to environmental change (Orsini *et al.* 2013; Franks *et al.* 2018).

An increasing number of studies have used this “resurrection approach” to examine rapid evolution to increased drought. Some of these studies convincingly showed that plants adapted their phenology towards an earlier flowering in order to avoid drought (Franks *et al.* 2007; Nevo *et al.* 2012; Vigouroux *et al.* 2011; Thomann *et al.* 2015). For growth traits, results appear to be more species-specific. For example, in an experiment with *Mimulus laciniatus* by Dickman (2016) the descendants were better adapted to drought and grew larger, whereas Vigouroux and colleagues (2011) found the opposite results in a study with *Pennisetum glaucum* where descendant populations that experienced drier climates during 27 years grew smaller. Thus, although species may vary in their evolutionary responses to drought, some traits show consistent evolution across species. Multi-species resurrection experiments can elucidate such commonalities, and therefore improve our ability to forecast future evolution under climate change. Nevertheless, such studies are still rare because they require seed collections specifically compiled for this purpose (Franks *et al.* 2018). In order to use untapped resources for environmental change research, seed collections stored in seed banks can be used in similar ways as resurrection studies. To conduct such historical comparisons, the amount of stored seeds should be high and information on the sampling locality, the number of collected individuals and the genetic diversity should be available.

Here, we used seed material from seed banks in historical comparisons to investigate whether single populations of multiple plant species from Mediterranean and temperate regions

of Europe have evolved their drought tolerance over the last decades in response to more frequent and longer drought events (Met Office 2011; DWD 2018; IRM 2020). To investigate this, we conducted two complementary common garden experiments in which we applied drought treatments to plants raised from seeds stored for at least 21 years in three different seed banks (ancestors) and from seeds that we collected from the same populations in 2018 (descendants). Seedling establishment is a key process for population survival (Grubb 1977), and seedlings are especially susceptible to drought (Moles and Westoby 2004). Therefore, the drought resistance of seedlings should be under high selection pressure in increasingly dry and more variable environments (Schupp 1995; Fenner and Kitajima 1999). In 2019, Dickman and colleagues already published a resurrection study with *Mimulus laciniatus* showing that contemporary populations, which experienced droughts during the last years, germinated earlier (Dickman *et al.* 2019). However, studies on evolution of drought resistance in early life stages are generally still scarce and multi-species experiments using watering treatments are missing. This is why we examined differences between ancestors and descendants in their responses to drought treatments at early life stages for multiple species.

As seedlings are generally very sensitive to dehydration, especially in environments with large fluctuations in water availability and high chance of drought events (Padilla and Pugnaire 2007), we expect evolutionary change in drought tolerance when drought regimes change. We used four herbaceous Mediterranean species in the first experiment to test whether seedlings of the descendants survived longer without watering than the seedlings of their ancestors ("seedling survival experiment"). In the second experiment ("watering response experiment") we worked with juvenile (i.e. establishing, non-flowering) plants from nine temperate European species which experience the lowest precipitation during early growth between April and June (data from the Climatic Research Unit; Camarillo-Naranjo *et al.* 2019; Harris *et al.* 2020). We subjected ancestors and descendants to well-watered vs. dry conditions and compared their growth responses within the first weeks after germination to test the hypothesis that populations evolved phenotypic traits, and/or their plasticities, to cope with increased droughts.

Material and methods

Seed collection

For the seedling survival experiment, we obtained seeds of four species – *Anthemis maritima, Matthiola tricuspidata, Medicago marina* and *Plantago subulata* – from the seed bank at the

"Conservatoire Botanique National Méditerranéen de Porquerolles" (CBNMed, Hyères, France). For the watering response experiment investigating juvenile plants, the seed material of eight species – *Centaurium erythraea*, *Clinopodium vulgare*, *Digitalis lutea*, *Leontodon hispidus*, *Melica ciliata*, *Pimpinella saxifraga*, *Sedum album* and *Teucrium chamaedrys* – was provided by the seed bank of "Meise Botanic Garden" (Belgium) and of one species – *Dianthus carthusianorum* – by the "Botanical Garden of the University of Osnabrück" (Germany). For both experiments, we only used seeds which had precise sampling dates and location records, which occurred in nature protection areas, and which had been stored for at least 21 years. We selected species with a short life cycle as they were expected to respond more quickly to selection and were therefore more likely to show rapid evolution. We confirmed under greenhouse conditions that all chosen species started to reproduce at least in the second year of growth (Table 1). To reduce the chance that the sampled populations were strongly influenced by gene flow from other populations, we specifically chose seed material from populations of origin that were relatively isolated (but sufficiently large).

Franks and colleagues recommend the following criteria for seed sampling in resurrections studies: at least two time points for sampling, each time collecting >30 plants while keeping maternal lines separated (Franks *et al.* 2018). These criteria safeguard that genetic diversity within a population is captured sufficiently and that the original genetic structure is kept largely intact. As the materials from the seed banks were not originally collected with the aim to conduct resurrection experiments (e.g. the number of sampled individuals is often unknown, and all sampled seeds were bulked) our study does not fulfil these strict criteria. However, with two types of further information, we are convinced that seed bank material can be used in a similar way to the resurrection approach, and that historical comparisons based on it are meaningful.

Table 1 (next page) Study species used in the two experiments with details on the amount of stored seeds in the seed banks, number of sampled individuals (2018), estimated population size (2018), year of maturity detected under greenhouse conditions and species' life-form, measured traits in the watering response experiment, number of seeds used, germination rates and number of replicates for each treatment and temporal origin within the experiments.

Species	Amount of stored seeds	Number of sampled individuals in 2018	Estimated population size in 2018	Year of first reproduction (greenhouse) and life-form	Plant size trait	Number of leaves or shoots	Number of used seeds and germination rate		Replicates
							Ancestors	Descendants	
A. maritima	1000	80	2500	2nd year (perennial)	-	-	100 (25%)	100 (33%)	30
M. tricuspidata	1000	15	30	1st year (annual)	-	-	100 (95%)	100 (98%)	30
M. marina	500	50	200	2nd year (perennial)	-	-	100 (45%)	100 (90%)	20
P. subulata	500	103	200	1st year (perennial)	-	-	100 (78%)	100 (29%)	9
C. erythraea	1000	20	100	2nd year (perennial)	diameter	leaves	200 (50%)	1000 (50%)	12
C. vulgare	1000	47	500	1st year (perennial)	height	shoots	200 (75%)	200 (90%)	12
D. carthusianorum	500	20	150	1st year (perennial)	diameter	leaves	100 (25%)	100 (50%)	7
D. lutea	2500	20	150	2nd year (perennial)	diameter	leaves	500 (20%)	500 (30%)	12
L. hispidus	1000	20	100	1st year (perennial)	diameter	leaves	300 (30%)	300 (80%)	12
M. ciliata	1000	21	25	1st year (perennial)	height	shoots	200 (75%)	150 (50%)	7
P. saxifraga	1000	20	75	2nd year (perennial)	diameter	shoots	200 (50%)	200 (25%)	8
S. album	1000	20	1000	2nd year (perennial)	diameter	shoots	500 (20%)	500 (20%)	12
T. chamaedrys	1300	20	100	2nd year (perennial)	height	shoots	200 (20%)	300 (20%)	12

The first type of evidence is information on sampling. All species occur rather abundantly in their original habitat, the amount of seeds within the stored lots was high (Table 1) and the collectors tried to maximize the number of sampled individuals (pers. comm. with the former collectors). Thus, we are confident that the genetic diversity of seed bank collections we used is representative of what was present at the time of sampling.

The second important information comes from a molecular analysis using ddRAD-SNP marker data for all species. We assessed pairwise genomic relatedness among samples within ancestral and descendant populations using two estimators, genomic relatedness *G* (Yang *et al.* 2010) and the kinship estimator r^{β} (Goudet *et al.* 2018), which were applied to ancestral and descendant individuals in one population. In addition, we assessed genomic diversity within ancestral and descendant populations as allelic richness, Ar (El Mousadik and Petit 1996). We show that the relatedness of plants is similar within ancestors and descendants for 10 out of 13 species, providing further support for similar sampling procedures and that sufficient seeds were sampled during both periods, avoiding biased sampling of particular mother plants. Furthermore, allelic richness was similar for 10 out 13 species, indicating low influence of bottlenecks or gene flow (see Box 2, p. 46–48).

For the seedling survival experiment, we used seeds of four Mediterranean species (Table 1): *A. maritima, M. tricuspidata, M. marina* and *P. subulata*. The seeds of these four species had been collected in the same area close to Hyères, Southern France, between 1992 and 1997. Data from the Climatic Research Unit (Camarillo-Naranjo *et al.* 2019; Harris *et al.* 2020) show that average temperatures between March and July have increased and precipitation decreased during the last 30 years in comparison to the long-term means from 1900–1999. Combining both environmental variables, we calculated the “De Martonne aridity index” (IDM, Pellicone *et al.* 2019), which demonstrated soaring drought during the last three decades ($IDM_{1988–2018}$ = 21.3) in comparison to the long term mean ($IDM_{1900–1999}$ = 25.5). In addition, precipitation variability (CV) during 2009–2018 was 35% larger than during 1988–1997 (Camarillo-Naranjo *et al.* 2019, Harris *et al.* 2020).

For the watering response experiment, we used seeds of nine temperate species (Table 1): *C. erythraea*, *C. vulgare*, *D. carthusianorum*, *D. lutea*, *L. hispidus*, *M. ciliata*, *P. saxifraga*, *S. album* and *T. chamaedrys*. The seeds of these nine species had been collected between 1992 and 1995 in Belgium (two different regions) and close to Osnabrück (Germany). Comparing the last 30 years with 1900–1999, the average temperatures between March and July have also increased and precipitation has decreased, which led to lower values of the “De Martonne aridity index” indicating a drier environment in all three regions. For *D. carthusianorum,* close to

Osnabrück we calculated $IDM_{1988–2018}$ = 31.4 compared to $IDM_{1900–1999}$ 34, for *L. hispidus* $IDM_{1988–2018}$ = 37.2 compared to $IDM_{1900–1999}$ = 40, and for the remaining species in Belgium $D_{1988–2018}$ = 36.1 compared to $IDM_{1900–1999}$ = 37.4. In addition, precipitation variability (CV) during 2009–2018 compared to 1988–1997 was 45% decreased for *D. carthusianorum*, did not change for *L. hispidus* and increased by 25% for the remaining species in Belgium.

To obtain the descendants, seeds of all species were collected from the same populations in spring (Mediterranean species) and summer (temperate species) of 2018. To be sure about the resampling of the same population, the exact location of all populations was identified either by precise coordinates of the target population, or by re-identification of the same collector as 30 years ago. In each population, we aimed to sample at least 30 individuals with different height and life stages to account for temporal variation in fruit ripening. The realized sample size ranged from 15 to 103 (Table 1) with only 15 individuals sampled from *M. tricuspidata* and 21 individuals from *M. ciliata* because population size of these species was only 30 and 25 individuals, respectively. We then bulked all seeds to have a comparable seed mix as for the ancestors.

Seedling survival experiment

For the seedling survival experiment with the Mediterranean plants, we initially sowed 100 seeds per temporal origin (i.e. ancestors and descendants) of each species, germinated them (for more Information see Box 1, p. 21) and recorded germination success every second day. The germination rates were similar for ancestor and descendant seeds of *A. maritima* and *M. tricuspidata* but they differed for *M. marina* and *P. subulata* (Table 1).

For each species we filled one seedling tray (96-cell QuickPot®, 3.8 × 3.8 cm cells) with a standard peat-free potting soil (Einheitserde®, BioLine, Topfsubstrat Öko torffrei) and planted seedlings (see Table 1 for the numbers of individuals) into every other cell so that the seedlings did not grow directly next to each other. We planted the ancestors and descendants in an alternating pattern. To identify the seedlings, we noted their positions but did not use any labels in order to reduce observer bias. The trays were placed in a walk-in growth chamber with a light/dark cycle of 12/12 hours and 23/18 °C (light intensity = 230 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 50% relative humidity). The seedlings were watered regularly for 2–3 weeks (depending on the species) to allow their establishment. After that, we stopped watering to simulate drought. We recorded mortality due to desiccation at least every second day. A seedling was scored as dead when it was completely dry and all leaves had lost their green colour. We cut each dead seedling 1 mm above ground, dried it at 60 °C for 72 h, and weighed it.

Watering response experiment

For the watering response experiment with temperate plant species, we germinated 100–1000 seeds per temporal origin in seedling trays (see Table 1 for precise numbers and Box 1 p. 21 for details). We kept the seedlings in these trays for three months before the start of the experiment.

For the main experiment, we filled 9 × 9 cm pots with a 3:1 mixture of peat-free potting soil (Einheitserde®, BioLine, Topfsubstrat Öko torffrei) and sand (0–2 mm play sand, WECO GmbH). In early June 2019, we transplanted each seedling into its own pot while making sure that we transplanted pairs of ancestors and descendants that were approximately of equal size. Right after transplantation, we measured shoot length or rosette diameter (henceforth referred to as plant size) as well as, depending on the species, the number of leaves or shoots (Table 1). After two weeks, we split all juvenile plants into a well-watered control group and a drought group, with 7–12 replicates per temporal origin and species (Table 1). When five of the pots of a species had a dry soil surface, all plants of that species were watered, with control plants receiving 60 mL and drought plants receiving 30 mL water at each watering. We re-randomized all pots in the greenhouse weekly. After eight weeks we repeated the growth trait measurements and then harvested all plants and determined their aboveground biomass after oven-drying at 60 °C for three days.

Statistical analyses

In both experiments, we used linear models to examine differences between the temporal origins (ancestors vs. descendants). We analysed data from the seedling survival experiment with models testing for effects on the number of days of survival (i.e. time between start of the drought treatment and death) and the aboveground biomass as response variable. In both models, we included species identity and its interaction with temporal origin as an additional explanatory variable to account for species differences. For the analysis of the number of days of survival we also included the number of days between transplanting and the start of the experiment as a covariate, and for the analyses of aboveground biomass we included the total lifespan of the seedling as a covariate. In addition to these multi-species models we also analysed the data separately for each species, using the same models but excluding species identity. Finally, we used linear models to test whether the aboveground biomass of a plant predicted its number of days of survival, while correcting for the total lifespan of the seedling by including it as a covariate.

We analysed the data from the watering response experiment with juvenile plants with models testing for effects of drought treatment on plant size, number of leaves of shoots, and aboveground biomass. We first square-root-transformed the number of leaves and aboveground

biomass to normalise model residuals. In order to be able to compare different measurements across the nine species, we standardised all data per trait to a mean of 0 and a standard deviation of 1. We then analysed the variation in plant size, number of leaves or shoots, and aboveground biomass with linear models that included temporal origin (ancestors vs. descendants), treatment (drought vs. control) and species, and all possible interactions, as explanatory variables. A two-way interaction between temporal origin and treatment would suggest that plants have evolved a different response to drought, and a three-way interaction between temporal origin, treatment and species would suggest that species vary in their evolutionary responses to drought. In addition to the multi-species analyses, we also analysed the data for each species separately, using linear models that included only temporal origin, treatment and their interaction. As the sizes of the transplanted seedlings differed, we corrected for this by including the initial size measurements as a covariate in all our models.

For all models, we visually checked the residuals for normality and heteroscedasticity. All analyses were done in R (Version 4.0.2) using the package *plyr* for data management (Wickham 2011) and the *lm()* function to run linear models.

Results

Seedling survival experiment

Across species, seedlings from descendants survived on average almost two days longer than seedlings from ancestors (Fig. 1A, $F_{1,208}$ = 12.99, p < 0.001). The studied species also differed in mean survival ability (Fig. 1A, $F_{3,208}$ = 255.21, p < 0.001) and we found an interaction between species and the temporal origins (Fig. 1A, $F_{3,208}$ = 2.74, p = 0.04). Although descendants of *Matthiola tricuspidata* and *Medicago marina* also survived slightly longer, the overall effect of temporal origin was mainly driven by one of the species, *Anthemis maritima*, since only descendants of this species showed a significantly longer survival than their ancestors in the individual-species analyses ($F_{1,60}$ = 6.01, p = 0.017).

Across species, seedlings from descendants had a significantly lower biomass than those from ancestors (Fig. 1B, $F_{1,204}$ = 19.92, p < 0.001). Again, there was an interaction between species and temporal origin (Fig. 1B, $F_{3,204}$ = 3.57, p = 0.015), with the overall effect largely driven by *A. maritima* as only this species showed a significant biomass difference between temporal origins in individual-species analyses (Fig. 1B, $F_{1,59}$ = 6.08, p = 0.016). Across species, plants with a lower biomass generally survived longer ($F_{1,200}$ = 12.43, p = <0.001, r^2=0.46). However, at

the species level we observed a significant negative correlation between biomass and survival only for *A. maritima* ($F_{4,58}$ = 4.03, p = 0.006, r^2=0.16), whereas for *M. tricuspidata* ($F_{3,59}$ = 4.01, p = 0.012, r^2=0.13) and *M. marina* ($F_{4,46}$ = 7.71, p <0.001, r^2=0.35) there were positive correlations, i.e. larger plants survived longer.

Watering response experiment

The drought treatment had a significant effect on all three measured growth traits. Across all nine species, plants grown under drought conditions were smaller, produced fewer branches or leaves and had a lower aboveground biomass (Fig. 2A–C, Table 2). These observations were also consistent at the species level: in all species where a significant effect occurred, drought decreased plant growth (Table 3). Seven out of the nine tested species were affected in at least one of the measured traits. The temporal origin did not affect plant size in any of the studied species, but we found a significant difference in the number of leaves or shoots and in aboveground biomass between the ancestors and descendants of two and three species, respectively. In *Centaurium erythraea* and *Melica ciliata,* descendants produced significantly more leaves or shoots and biomass, but in *Dianthus carthusianorum* descendants produced less biomass (Table 3). Across species, there was a significant drought-by-temporal origin interaction for plant size (Fig. 2A, Table 2). While ancestral plants showed only a slight decrease of plant size in response to drought, the descendants strongly decreased plant size under drought. This observation is consistent across species, as there was no significant three-way interaction among the watering treatment, temporal origin and species in our model (Table 2). However, none of the individual-species models showed a significant treatment by temporal origin interaction for plant size (Table 3).

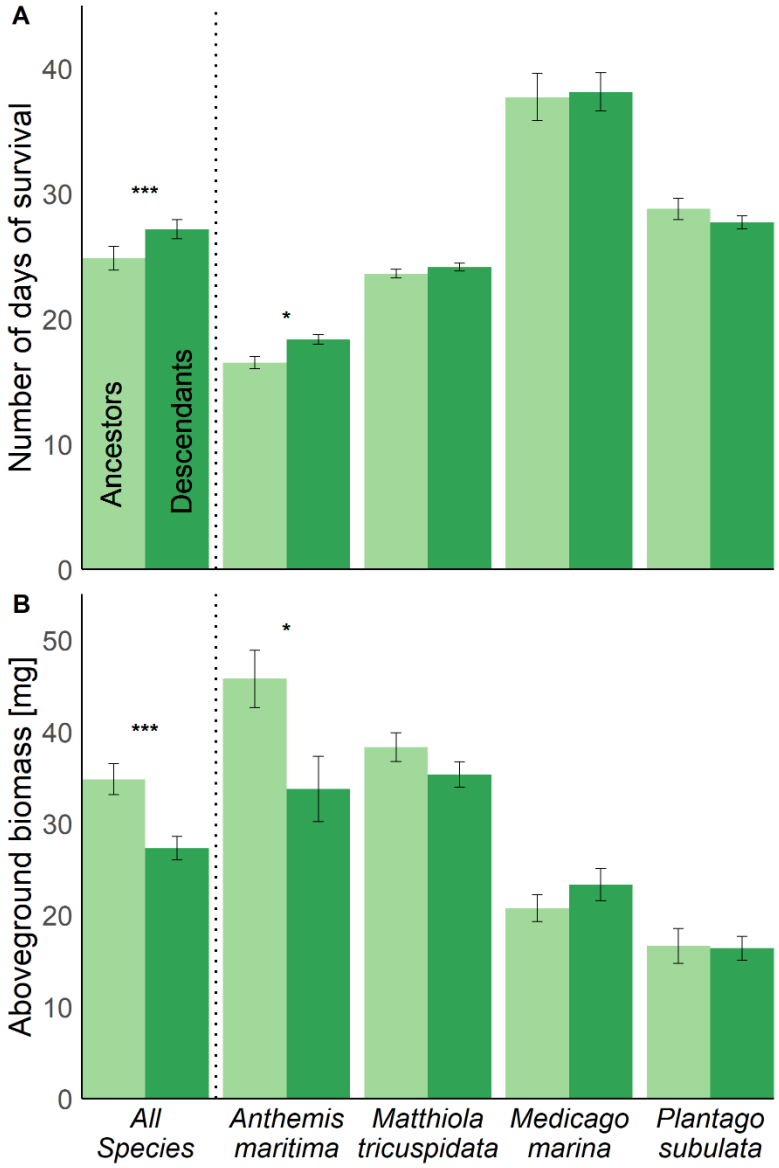

Figure 1 Mean number of days of survival after watering ceased (A) and aboveground biomass at harvest (B) of seedlings of four Mediterranean species *Anthemis maritima, Matthiola tricuspidata, Medicago marina, Plantago subulata* from two different temporal origins (ancestors vs. descendants). The bars show means and standard errors. * = $p<0.05$, *** = $p<0.001$

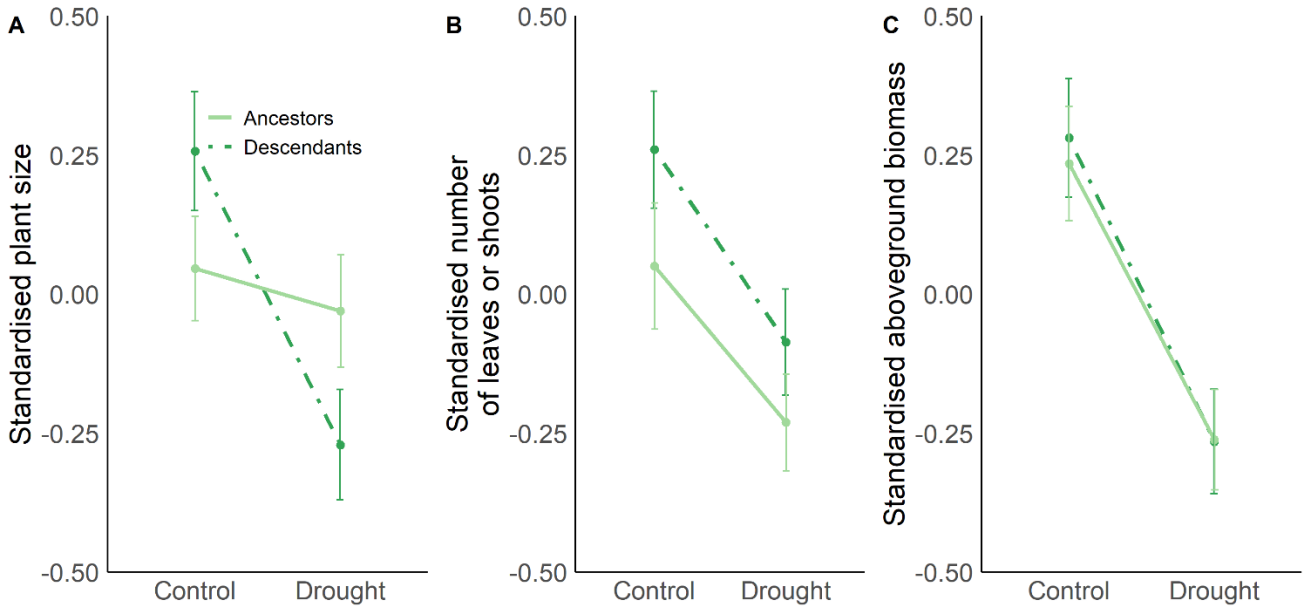

Figure 2 Reaction norm plots of plant size (A), number of leaves or shoots (B) and aboveground biomass (C) in the watering response experiment. The data are transformed and averaged across all nine species from two temporal origins (ancestors vs. descendants). Error bars show standard errors.

Table 2 *F*- and *p*-values from cross-species linear models of the watering response experiment, each testing for effects of species, treatment (drought vs. control), temporal origin (ancestors vs. descendants), and their interactions. The arrows indicate the direction of a significant effect (↓/↑ = transformed values of the descendants or drought, respectively, are smaller/larger). Significant results are bold marked. Degrees of freedom (d.F.) are shown for tested variables and vary for the residuals.

		Plant size		**Number of leaves or shoots**		**Aboveground biomass**	
	d.F.	*F*	*p*	*F*	*p*	*F*	*p*
Species	8	**19.92**	**<0.001**	**3.08**	**0.01**	**13.71**	**<0.001**
Treatment	1	**14.88 ↓**	**<0.001**	**10.56 ↓**	**<0.001**	**41.90 ↓**	**<0.001**
Origin	1	<0.01	0.97	3.60	0.06	0.25	0.62
Species × Origin	8	0.71	0.68	**2.38**	**0.02**	**4.27**	**<0.001**
Species × Treatment	8	1.91	0.06	1.28	0.26	**2.63**	**0.01**
Treatment × Origin	1	**6.16**	**0.01**	0.05	0.83	0.01	0.91
Species × Treatment × Treatment	1	0.48	0.87	0.82	0.58	1.47	0.33

Table 3 *F*- and *p*-values from linear models of the watering response experiment, each testing for effects of treatment (T; drought vs. control), temporal origin (O; ancestors vs. descendants), and their interaction (T × O). The arrows indicate the direction of a significant effect (↓/↑ = transformed values of the descendants or drought, respectively, are smaller/larger). Significant results are bold marked. Degrees of freedom for all tested effects was 1 and varies for the residuals.

Species	Plant size						Number of leaves or shoots						Aboveground biomass					
	T		O		T × O		T		O		T × O		T		O		T × O	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
C. erythraea	**7.91** ↓	**0.01**	3.69	0.06	4.04	0.05	0.01	0.94	**4.22** ↑	**0.04**	0.17	0.68	**5.50** ↓	**0.02**	**15.40** ↑	**<0.001**	2.87	0.14
C. vulgare	0.28	0.60	0.05	0.82	0.67	0.42	1.07	0.31	0.62	0.44	0.61	0.44	0.65	0.43	0.09	0.77	0.67	0.42
D. carthusianorum	0.25	0.62	0.11	0.75	0.47	0.47	1.19	0.29	0.51	0.48	0.51	0.48	0.07	0.79	**10.23** ↓	**0.01**	**4.92**	**0.04**
D. lutea	0.73	0.40	0.04	0.85	0.04	0.85	**7.38** ↓	**0.01**	2.74	0.11	0.39	0.54	**5.12**↓	**0.03**	<0.01	0.95	0.21	0.65
L. hispidus	**20.25**↓	**<0.001**	1.64	0.21	0.86	0.36	**7.26** ↓	**0.01**	3.13	0.08	<0.01	0.98	**29.44** ↓	**<0.001**	3.86	0.06	0.81	0.37
M. ciliata	0.36	0.56	0.19	0.67	2.43	0.13	1.55	0.23	**9.58** ↑	**0.01**	0.56	0.46	2.77	0.11	**23.24** ↑	**<0.001**	0.60	0.45
P. saxifraga	1.50	0.23	2.54	0.12	0.48	0.50	**5.54**↓	**0.03**	1.55	0.22	0.10	0.76	4.12	0.05	0.16	0.69	2.24	0.15
S. album	**5.01**↓	**0.03**	0.04	0.84	0.65	0.43	**12.23** ↓	**<0.001**	0.61	0.44	0.90	0.35	**37.07** ↓	**<0.001**	0.23	0.63	0.73	0.40
T. chamaedrys	1.75	0.19	0.39	0.53	0.97	0.33	1.34	0.25	1.62	0.21	0.92	0.34	3.02	0.09	2.63	0.11	0.88	0.35

Discussion

We used seed material stored in seed banks and contemporary seeds collected from the same populations several decades later to investigate rapid evolution. Specifically, to test for recent evolutionary responses of plants in early life stages to climate change, we compared the drought tolerance of ancestral and descendent plants of several Mediterranean (seedling survival) and temperate plant species (juvenile plants). In the species' regions of origin, drought is a particular stress during the investigated life stages.

Seedling survival experiment

In our seedling survival experiment with Mediterranean plant species, we found that in one of the four studied species, *Anthemis maritima*, descendant seedlings survived longer under drought than their ancestors and produced less aboveground biomass (Fig. 1). Although our experimental approach cannot assert whether observed evolutionary changes are adaptive, our observations are consistent with what would be expected if adaptation to drought had occurred in the studied population during the last decades.

Survival under drought can be enhanced by a small plant size, as we observed for *A. maritima*. In a multi-species approach Harrison and LaForgia (2019) compared seedling survival of ten grassland herbs under different water availability. They showed that the survival rate of smaller seedlings was higher under dry conditions. A possible explanation for this is reduced evapotranspiration through decreased leaf number, leaf size and branching and lower plant biomass (Aroca 2012). These observations also fit to the observation that plants in dry conditions often decrease aboveground biomass production and allocate more biomass to roots, leading to a higher root:shoot ratio (Martin and Stephens 2006; Villagra and Cavagnaro 2006; Erice *et al.* 2007). However, increased seedling drought tolerance can also be mediated by other traits such as root structures (e.g. hypocotyl hairs; Aronne and De Micco 2004). However, it is also possible that the observed reduction in plant size was the result of passive stress responses instead of the above-mentioned active responses of plants to droughts.

Our main research question was to test for evolutionary changes between ancestors and descendants, and our historical comparison, a somewhat less strict version of the resurrection approach, has some weaknesses here, particularly when interpreting biomass results. First, we did not grow a "refresher generation" of ancestors and descendants prior to our main experiment, because part of the study species only started to reproduce in the second year. We therefore cannot exclude that storage or maternal effects influenced the results (Franks *et al.* 2018). Second, if stored seeds have low germination rates, there is a possibility of invisible fractions

(Weis 2018), with germinating individuals representing only a subset of the stored phenotypes. In *A. maritima* the germination rate for the ancestors was only 25%, so we cannot rule out such invisible fraction effects. A third potential drawback of such historical comparisons with seed bank material not designed for these purposes is that sampling efforts can be very different for seeds from different periods. Fortunately, our molecular analysis found similar levels of relatedness among ancestors and descendants, indicating that the sampling probably has been conducted in a similar way and that sampling effort was sufficiently high.

While descendants of *A. maritima* showed improved drought resistance compared to their ancestors, we did not find similar patterns for three other species (Fig. 1A,B). Possible reasons for this could be that these species have not evolved due to lack of genetic variation or other evolutionary constraints (e.g. trade-offs) preventing evolution in specific phenotypes. Alternatively, it is also possible that these species evolved different (phenological) strategies to cope with drought during the seedling stage which we did not explicitly study.

In summary, we show that seedling survival under drought has likely evolved in the last decades through adjustments of phenology and growth strategy in one out of four studied Mediterranean plant species. To disentangle evolution by means of natural selection from random evolutionary processes, i.e. mutation, drift and gene flow, quantitative genetic differentiation (Q_{ST}) can be compared with neutral molecular differentiation (F_{ST}) (Merilä and Crnokrak 2001; McKay and Latta 2002). Unfortunately, our design, comparing one ancestral with one descendant population per species, is suboptimal for such comparisons. However, our molecular data suggests similiarty in the genetic background of two species accompanied by some uncertainties for *M. tricuspdiata* and *P. crassifolia* (see Box 2, p. 46–48). Furthermore, future experiments could exclude potential influences such as maternal or storage effects by growing refresher generations (Franks *et al.* 2018).

Watering response experiment

In our watering response experiment with nine species from temperate Europe, we subjected juvenile plants to drought that generally led to decreased plant size, number of leaves or shoots and aboveground biomass. Across species, we found no differences in mean traits between ancestors and descendants, but there was an overall difference between ancestors and descendants in the plasticity of plant size in response to drought, with a much stronger decrease of size in the descendant plants (Table 2). Since precipitation variability has increased for most of the studied species during the last decades this observation could corroborate predictions that such conditions favour the evolution of increased phenotypic plasticity (Sultan and Spencer 2002;

Alpert and Simms 2002; Gianoli and Valladares 2012).

In none of the single-species analyses did we find an interaction between treatment and temporal origin for plant size, which is probably partly explained by the moderate replicate numbers per species. Nevertheless, seven out of nine species showed the same trend for plant size as the cross-species analyses (Fig. S1), and the three-way-interaction with species, temporal origin and treatment was insignificant, indicating similar cross-species patterns (Table 2). Since plant biomass and number of leaves or shoots were unaffected, this stronger shift in plant size under dry conditions could be accompanied by changes to other functional traits we did not measure in our study such as leaf thickness or leaf shape, which are known to be highly plastic (Gianoli and Gonzáles-Teuber 2005; Lázaro-Nogal *et al.* 2015). A reduction of leaf area accompanied by increasing leaf thickness and/or more pubescent leaves may reduce evapotranspiration (Gianoli and Gonzáles-Teuber 2005) and can therefore be a successful strategy under drought (La Riva *et al.* 2016).

Plant responses to drought are generally complex and may even differ between closely related species (Bouzid *et al.* 2019), as drought affects plants at various developmental stages and in different tissues (Yordanov *et al.* 2000). Our experiment does not allow us to – but future studies should – identify the processes underlying the observed patterns that may include increased resource allocation to roots (Martin and Stephens 2006; Villagra and Cavagnaro 2006; Erice *et al.* 2007), changes in stomatal density (Liu *et al.* 2015) and reduced evapotranspiration (Aroca 2012), or a combination of these and other factors.

We also found significantly larger plant sizes but not higher aboveground biomasses in the control treatment for the descendants compared to ancestors across species. This may be an adaptation of the species' life cycles: As flowering onset is often related to plant size (Vile *et al.* 2006; Sun and Frelich 2011), we argue that plants grow and develop fast when water supply is sufficient to escape potential drought stress later in their life cycle (Grene *et al.* 2011). When interpreting the results of our study we should keep in mind that our drought treatment was simplified, with water applied at constant low versus constant normal levels. In nature, patterns of water availability may be more variable, and we do not know how our plants would have, for example responded to drought after a period of sufficient watering. This is important given that under ongoing climate change, not only mean precipitation but also temporal patterns are changing.

Greater environmental heterogeneity in space or time, when perceived within the organism's – or its immediate descendants' – lifetime, is generally expected to favour greater phenotypic plasticity (Alpert and Simms 2002; Bradshaw and Holzapfel 2006; Matesanz *et al.*

2010). In the regions of origin of most of the study species, drought frequency has increased over the last 20 years (Spinoni *et al.* 2018), and environmental conditions have thus became more unpredictable (Altvater *et al.* 2011). This could have favoured evolution of stronger plasticity through natural selection for more plastic genotypes (Ackerly *et al.* 2000; Richards *et al.* 2006). To test whether the observed greater plasticity in plant size of the descendant plants is an adaptive change requires further experiments that include longer-term measurements of plant fitness (Richards *et al.* 2006). Ideally, such experiments should take place at the species' sites of origin and incorporate a large number of populations which experienced different rates of climate change, and in particular increased precipitation variability, during the past decades.

Although our results may be influenced by other factors, we are confident that we observed a true evolutionary pattern here that is common in nature: greater plasticity of descendants – as a trend – was consistent for seven out of the nine studied species (Fig. S1), which is very unlikely if part or all of these patterns were due to chance or unintentional selection during sampling and storage or due to maternal effects on each species separately (see first discussion section above). Furthermore, germination rates in most species were high, and there were no relationships between germination rate and plasticity, suggesting that variation in germination rates did not affect other traits. However, we cannot completely exclude potential storage effects or hidden fractions, especially for *Leontodon hispidus*, for which germination rates differed strongly between ancestors and descendants (Table 1). Random evolutionary processes, such as drift or gene flow, as well as unintentional selection are unlikely to have stronger effects than those exerted by the drought treatments, which pose strong selection pressures on seedling recruitment and drought responses (Schupp 1995; Fenner and Kitajima 1999).

Using seed bank material for historical comparisons

Resurrection studies are a powerful tool for studying recent evolution, but the appropriate genetic resources are rarely available. Large-scale long-term efforts have recently been set up to conduct powerful resurrection studies in the future (e.g. "Project Baseline", Etterson *et al.* 2016). However, if material from regular seed banks could be used for similar before-after comparisons, it would open up a vast resource for environmental change research. Although seed banks often lack population replicates within species, multi-species approaches can make studies more powerful by testing for common evolutionary patterns across taxa.

Despite previously mentioned shortcomings of our study, we show that it is possible to use seed bank material, not explicitly collected for resurrection studies, for similar historical comparisons. In our study, genomic relatedness analyses indicated that the ancestor and

descendant seed pools were similar, and that seed sampling has been conducted in a comparable way. Our molecular data also suggests that the genetic diversity of seed bank collections and newly collected seeds was sufficiently large for conducting the experiments. We are therefore reasonably confident that the use of seed bank material in our study was meaningful. Our approach opens up a new avenue for studies on recent plant evolution and may be a useful complement to other approaches that study contemporary populations or use other stored materials such as herbarium specimen (DeLeo *et al.* 2019; Lang *et al.* 2019).

Conclusions

Ongoing climate change is expected to influence the evolution of plant populations, but so far experimental tests of this are rare. Our multi-species historical comparisons using taxa from two different biogeographic regions in Europe investigating drought responses of plants in early life stages indicate that plants have evolved within the last decades, possibly in response to increased drought frequencies. We observed evolutionary changes in several, but not all, species, in both trait means and trait plasticity in response to experimental drought. Given the increased occurrence of drought events in most of the populations of origin, our results suggest that climate change may have already influenced the evolutionary trajectory of many plant species in different regions of Europe. Our study also demonstrates that historical comparisons similar to the resurrection approach can be made using plants from seed bank collections, and are a powerful tool for studying rapid evolution in plants. There is great potential for future studies to use the wealth of seed bank collections for investigating rapid adaptation to recent environmental changes. Replicated populations of the same species may be scarce in seed banks, which is why a multi-species approach is generally advantageous. Ideally, seeds from a refresher generation should be used to minimize possible maternal effects. To disentangle adaptive from non-adaptive and maladaptive responses to recent climate change, future experiments should incorporate fitness measures, comparative transplantations of descendants and ancestors into their original habitat. In addition, Q_{ST}-F_{ST} comparisons might help to infer the relative roles of selection and random evolutionary processes for population differentiation.

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Supplementary material

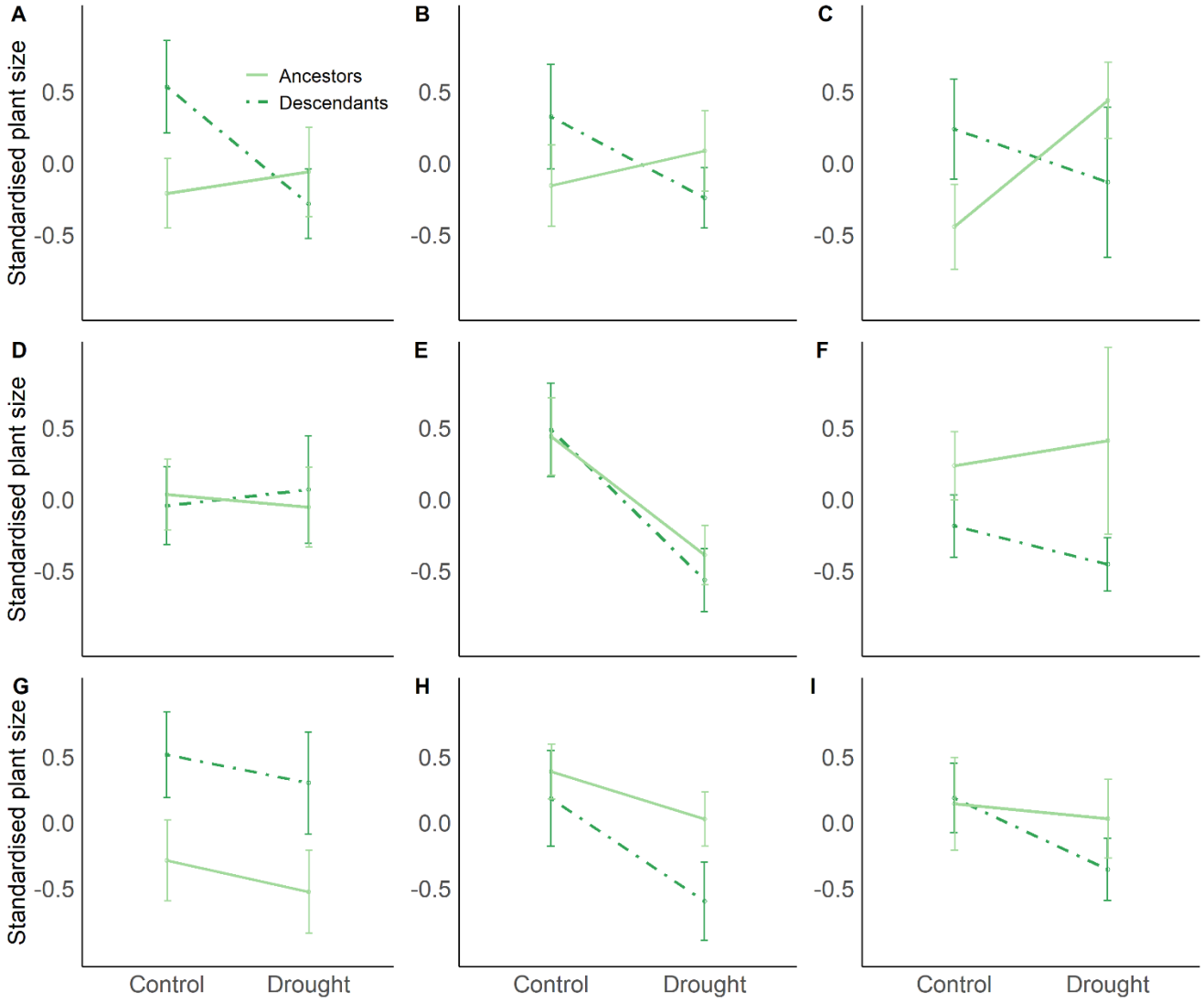

Figure S1 Reaction norm plots of plant size to drought. The data are transformed and separated for all nine tested species from two temporal origins (ancestors vs. descendants). Error bars show standard errors. A – *Centaurium erythraea*, B – *Clinopodium vulgare*, C – *Dianthus carthusianorum*, D – *Digitalis lutea*, E – *Leontodon hispidus*, F – *Melica ciliata*, G – *Pimpinella saxifraga*, H – *Sedum album*, I – *Teucrium chamaedrys*

Chapter III

Evolution of drought strategies and herbivore resistance after two decades of climate change in European plants

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Abstract

During the last decades, ongoing global warming coupled with increased drought frequencies has exerted increasing environmental stress on plant populations. Together with other, possibly interacting, biotic drivers, such as changes in insect herbivory, this may have resulted in complex evolutionary adaptation. The resurrection approach, comparing ancestors raised from stored seeds with their contemporary descendants under common conditions, is a powerful method to test for recent evolution in plant populations. We used 21-26-year-old seeds of four European plant species – *Matthiola tricuspidata*, *Plantago crassifolia*, *Clinopodium vulgare* and *Leontodon hispidus* – stored in seed banks together with re-collected seeds from their wild populations. To test for evolutionary changes, we conducted a greenhouse experiment that quantified heritable changes in plant responses to drought and simulated insect herbivory.

In three out of the four studied species, we found evidence that descendant populations evolved shorter life cycles through faster growth and flowering, possibly to escape summer droughts and potential insect outbreaks. Shifts in the osmotic potential and leaf dry matter content indicated that descendants also evolved increased drought resistance. A comparison of Q_{ST} vs. F_{ST} values, using ddRAD genotyping data, suggested that directional selection, and therefore adaptive evolution, was underlying some of the observed phenotypic changes. In summary, our study reveals evolutionary changes in plant populations over the last decades that are consistent with adaptation of drought escape and tolerance as well as herbivory avoidance, and it demonstrates the rapid and complex responses of European plants to recent environmental changes.

Introduction

Global change involves multiple abiotic and biotic changes that have affected European ecosystems over the last decades (Vitousek 1992; Matesanz *et al.* 2010; IPCC 2018). For plant populations, climate change is particularly challenging as it includes both increased temperatures and changes in precipitation (IPCC 2021). Their interaction can lead to an increased frequency and duration of drought events, as is for instance the case in Southern and Central Europe (Ruosteenoja *et al.* 2018; Samaniego *et al.* 2018; Spinoni *et al.* 2018). Under current scenarios, such novel conditions pose significant challenges to plant persistence (Shaw and Etterson 2012; Fleta-Soriano and Munné-Bosch 2016), and many plant populations are under increased risk of local extinction (Thomas *et al.* 2004; Urban 2015). Plant populations are already responding to environmental changes through migration (Parmesan and Yohe 2003; Lenoir *et al.* 2008), phenotypic plasticity and adaptive evolution (Holt 1990; Hoffmann and Sgrò 2011; Franks *et al.*, 2014).

A powerful method to test for recent evolution is the resurrection approach in which ancestors raised from stored seeds (e.g. from seed banks) are compared in common garden experiments to newly sampled descendants from the same populations (Franks *et al.* 2007; 2008; Orsini *et al.* 2013; Merilä and Hendry 2014; Franks *et al.* 2018). On its own, the resurrection approach only reveals whether evolutionary changes occurred; it cannot answer to which degree these resulted from natural selection, genetic drift, immigration of new genotypes, or mutations (Niklas 1997; Leinonen *et al.* 2008). However, with additional data from neutral molecular markers, comparisons between the neutral molecular differentiation (F_{ST}) and quantitative genetic differentiation (Q_{ST}) of phenotypic characters can help to better understand the importance of selective versus random evolutionary forces (Merilä and Crnokrak 2001; McKay and Latta 2002). Over the last years, some resurrection studies have already demonstrated rapid evolution of plants in response to climate change, including adaptation to increased drought intensities and frequencies, e.g. through shifts in flowering onset and growth (Franks *et al.* 2007; Vigouroux *et al.* 2011; Nevo *et al.* 2012; Thomann *et al.* 2015; Dickman 2016).

Besides the impact of climatic changes, another important stress and driver of evolutionary changes in plants is insect herbivory. The dynamics of invertebrate herbivory is also strongly affected by climate change (Futuyma and Agrawal 2009; Turcotte *et al.* 2014), as increased temperature enhances winter survival, growth and reproduction of insects as well as advancing and extending their annual life-cycle (Bale *et al.* 2002; Hamann *et al.* 2021). However, how changes in temperature and precipitation interact with insect herbivory in their effects on plants is

still not well understood (Schoonhoven *et al.* 2005; Prasch and Sonnewald 2013; Pandey *et al.* 2017; Descombes *et al.* 2020). The advancement of flowering as a response to climate change may also link to plant strategies for escaping from increased insect herbivory in summer due to enhanced growth and reproduction (Pilson 2000; *Bale et al.* 2002; Kawagoe and Kudoh 2010). For instance, Kawagoe and Kudoh (2010) found that an *Arabidopsis halleri* population with intensive floral herbivory advanced flowering in comparison to a population without herbivory, so responses may be synergistic under climate change. In contrast, Pilson (2000) found for *Helianthus annuus* delayed flowering is favoured under late season seed predation by insects, showing that plant responses depending on the temporal abundance of the insects. However, this observation indicates a possible trade-off between adaptations to herbivory and climate change as late flowering individuals would suffer more under summer drought.

Synergisms or trade-offs between adaptations to climate change and insect herbivory can also be expected for strategies of herbivore avoidance. For instance, under drought plants may reduce metabolically costly investment into chemical defences (Purrington 2000; Strauss *et al.* 2002; Jander 2018), which could make insect outbreaks particularly detrimental for plant populations already impacted by climate change (Haugen *et al.* 2008; Gutbrodt *et al.* 2011).
Although the climate change adaptation of plants has been studied intensively during the last years, we know of only one resurrection study that focused on evolution of herbivory defences (Bustos-Segura *et al.* 2014) and none that addressed interactions between climate change adaptation and adaptation to herbivory. We attempted to do this in our study, and employed the resurrection approach to test whether individual populations of four plant species underwent evolutionary changes in their drought responses over the last two decades, and whether there were simultaneous evolutionary changes in their responses to insect herbivory. To broaden the climatic scope of our study we included two species from the Mediterranean coastal habitat and two from temperate European grasslands, i.e. both from regions where temperatures have been increasing and where herbivory plays an important role (Moles *et al.* 2011; Kozlov *et al.* 2015). We modified the “resurrection approach” (Franks *et al.* 2018) by using seed collections stored in seed banks as an untapped resource for climate change research (Everingham *et al.* 2021; Rauschkolb *et al.* 2022). A potential drawback of using seed bank material for resurrection studies is that information on how and where the sampling took place in the past may be missing or insufficient. However, in our study we obtained a high amount of stored seeds, and we had ample information on the sampling locality, on the number of collected individuals and on the genetic diversity.

We grew ancestor and descendant lines in a common environment and subjected the plants to a full-factorial combination of drought and simulated herbivory. We further employed ddRAD genotyping data to compare F_{ST} and Q_{ST} values between ancestors and descendants in order to understand the adaptive significance of observed phenotypic changes.

Specifically, we addressed the following questions: (1) Do ancestral and contemporary populations differ in their phenotypes? (2) Does this differentiation result from selective or random processes? (3) Do ancestral and contemporary populations differ in their responses to drought and simulated herbivory, and if yes, are there synergies or trade-offs between these two types of responses?

Material and Methods

Study Species and seed origin

We investigated four plant species, *Matthiola tricuspidata* (L.) R.Br. (Brassicaceae) and *Plantago crassifolia* Forssk. (Plantaginaceae) from the French Mediterranean coast and *Clinopodium vulgare* L. (Lamiaceae) and *Leontodon hispidus* L. (Asteraceae) from temperate Belgian grasslands (Table 1). The two Mediterranean species are halophytic herbaceous species originating from sandy beaches; *M. tricuspidata* is an insect-pollinated annual and *P. crassifolia* a wind-pollinated perennial. The two temperate species are both insect-pollinated perennial forbs and typically found on dry calcareous soils. Although *L. hispidus* occurs mainly in dry grasslands and *C. vulgare* prefers thermophile woodland margins, both study populations originated from calcareous grasslands prone to drought. The perennial study species are all hemicryptophytes and reached maturity under greenhouse conditions within the first year of cultivation; all species except *L. hispidus* are self-compatible.

The source populations of all species underwent significant climate changes during the last decades, in particular temperature increases and precipitation decreases. For the Mediterranean species, average temperatures in March-July have increased by 1.1 °C, and precipitation anomalies summed to a decrease of around 1.5 mm per year in 1985–2020 compared to 1900–1999. In Belgium average temperatures have increased by approximately 0.9 °C in the area of *C. vulgare* and by 1.1 °C in the area of *L. hispidus*, and precipitation in spring and summer summed to a decrease of 29 mm per year when comparing the period 1985–2020 with 1900–1999 (data from CRU; Camarillo-Naranjo *et al.* 2019; Harris *et al.* 2020).

For all four species, we collected seeds in 2018 from the same wild populations as the seed collectors did >20 years ago for the seed bank collections. For the original seed bank

collections (1992-1997, depending on the species; see Table 1), large numbers of seeds of a representative number of individuals were collected in the populations and bulked, dried and stored at 5°C (Mediterranean species) or at -20°C (temperate species). We obtained the stored (ancestral) seeds from the seed banks at the Conservatoire Botanique National Méditerranéen de Porquerolles (CBNMed, Hyères, France) and at Meise Botanic Garden (Belgium). For the descendants, we re-collected seeds from all populations in the spring (Mediterranean species) and summer (temperate species) of 2018. We sampled 10–47 individuals per population (Table S1) and bulked their seeds to have a seed mix comparable to that of the ancestors.

Table 1 Study species used in the experiment with information on plant family, ancestor collection year, locality, germination rates, number of individuals at the start and the end of the experiment

Species	Family	Collection year, Location (City, Latitude, Longitude)	Germination rates		Number of plants at start (end) of the experiment	
			Ancestors	Descendants	Ancestors	Descendants
Matthiola tricuspidata	Brassicaceae	1994, Hyères (France), 43.044977, 6.132747	73%	84%	386 (400)	393 (400)
Plantago crassifolia	Plantaginaceae	1997, Hyères (France), 43.044977, 6.132747	96%	88%	396 (400)	398 (400)
Clinopodium vulgare	Lamiaceae	1992, Couvin (Belgium), 50.065255, 4.443902	42%	40%	281 (289)	251 (252)
Leontodon hispidus	Asteraceae	1995, Bassenge (Belgium), 50.792744, 5.672979	59%	77%	179 (200)	189 (200)

Although the seed bank material we used was not collected to perform resurrection experiments in the future, they can still be used for this purpose, for several reasons: (1) we know that the previous collectors aimed to maximize the number of sampled individuals (pers. comm.) (2) the numbers of seeds stored in the seed bank lots were high (>800; Table S1), which together means

that the risks of bottleneck effects should be low. Moreover, (3) analyses of single nucleotide polymorphism (SNP) markers showed similar levels of relatedness among ancestors vs. among descendants in three study species (*P. crassifolia*, *C. vulgare* and *L. hispidus*), which further supports the idea that sampling procedures were similar and thus the samples equally representative of these studied populations in both sampling periods (see Box 2, p. 46–48). Only for *M. tricuspidata* did we observe an increased level of relatedness in the descendants accompanied by a loss of alleles compared to the ancestors, which questions the comparability of the ancestors and the descendants as sampling may have been strongly different or unknown environmental factors may have directly affected the natural population over time between the two samplings (see Box 2 and Chapter I).

To disentangle evolutionary changes from possible storage and maternal effects (Franks *et al.* 2008), we cultivated a refresher generation prior to the main experiment in spring 2019 in a greenhouse at the University of Tübingen. For this, we first dark-stratified ancestor and descendant seeds of the Mediterranean species at 5°C for one week and of the temperate species for two months. For each species and temporal origin, we used 100–300 seeds, and we observed germination rates of at least 29% (Table S1). We transplanted 15 seedlings per temporal origin into 9 × 9 × 9 cm pots filled with a 1:3 mixture of sand (0–2 mm play sand, WECO GmbH) and potting soil (Einheitserde®, BioLine, Topfsubstrat Öko torffrei). The greenhouse was set to a light/dark cycle of 12/12 hours and temperatures of 20/15 °C as upper/lower limits. To prevent unintentional cross-pollination between ancestors and descendants, we grew the plants in net cages and hand-pollinated the plants within temporal origins, with random crosses within each set of 15 individuals. From these plants, we then harvested the ripe seeds for use in the subsequent experiments.

Experimental design

In spring and summer 2020, we conducted a common garden experiment, using the seeds from the refresher generation, in the same greenhouse and with the same climatic settings as above. Following the natural phenology of the species, we split the experiment into two parts: an experiment with the two Mediterranean species from January to April 2020 and an identical experiment with the two temperate species from May to August 2020. For the F2 experiments, we used ten seed families (i.e. maternal lines) per temporal origin for *M. tricuspidata* and *P. crassifolia,* nine ancestor and seven descendant seed families for *C. vulgare*, and five seed families for ancestors and descendants of *L. hispidus*. The numbers of seed families were lower than in the refresher generation because the pollination rates of some mother plants were too low

to produce sufficient seeds. After one week of dark-stratification at 5°C, we germinated 100 seeds per seed family in 54-cell QuickPot® trays filled with germination soil (Einheitserde®, BioLine, Pikiersubstrat). All germination rates were >40% and did not differ substantially between ancestors and descendants (Table 1). We transplanted 24–40 seedlings per seed family into 9 × 9 × 9 cm pots with a 1:3 mixture of sand (0–2 mm play sand, WECO GmbH) and potting soil (Einheitserde®, BioLine, Topfsubstrat Öko torffrei). After two weeks of seedling establishment, we randomly assigned 6–10 replicates per seed family to each of four treatment combinations: control, drought, herbivory, or drought plus herbivory. The watering treatments were as follows: the control plants were watered twice a week, with 100mL in weeks 3–6, 150mL in weeks 6–7 and to 200 mL in weeks 7–13. The drought plants received only half of the amount of water as the control plants throughout the experiment. Herbivory was simulated by clipping three holes in one leaf using a standardized hole puncher and pouring 15 µL jasmonic acid solution (1 mM) over this leaf (van Kleunen *et al.* 2004). The control group did not receive physical damage and was treated with a solution of the solvents (water and methanol) without jasmonic acid. The herbivory treatment was applied twice, three and five weeks after seedling establishment. We ran the experiment until >80% of the individuals of each species and temporal origin had flowered (10–13 weeks after transplanting).

Measurements

Two weeks after seedling establishment, and before the first treatments were applied, we estimated initial plant size as a covariate through vertical top-down photographs of all pots in a standardised photo box using a high-resolution digital camera. The amounts of green pixels per picture, calculated with a custom script in Python, were used as estimates of plant size. Throughout the experiment, we recorded the flowering of plants as the days when the first open flowers (*M. tricuspidata*, *C. vulgare*, *L. hispidus*) or anthers (*P. crassifolia*) were visible. To assess resource investment into aboveground biomass at the time of flowering we measured plant height for *M. tricuspdiata* and *C. vulgare*, the length of the longest leaf for *P. crassifolia*, and the rosette diameter for *L. hispidus*. From week 10, we successively harvested the plants separately by species, with one week of harvesting for each, and random order of harvesting within species.

In addition to these morphological and phenological characteristics, we also estimated two functional leaf traits, dry matter content (LDMC) and osmotic potential. To obtain fully hydrated leaves for this, we watered the pots and covered them with plastic bags overnight prior to harvesting. On the next day we weighed the fresh biomass of one randomly selected, well-developed leaf for LDMC, and we took an additional leaf of the same size from five replicate plants

per treatment and seed family for osmotic potential analyses, following the protocol of Májeková et al. (2019). We counted the numbers of inflorescences of each plant, and separated vegetative aboveground biomass and reproductive biomass. We then dried all biomass samples for three days at 60°C and determined the dry weight of each. We calculated LDMC by dividing the dry biomass of the target leaf by its fresh biomass. The dry weight of this leaf was added to calculate the total vegetative aboveground biomass (mg), and the reproductive investment as the fraction of the reproductive biomass to the total aboveground biomass. The samples for the osmotic potential analyses were kept frozen at -20°C until February 2021, when we determined the osmotic potential at full hydration ("osmotic potential" hereafter) using a Vapro5600 osmometer (ELITechGroup Benelux, Zottegem, Belgium).

Statistical analyses

For all statistical analyses we square-root transformed initial plant size, aboveground vegetative biomass and reproductive biomass in order to improve normality and homoscedasticity of the model residuals. We analysed the variation in the following nine variables for each species separately (number of finally used replicates in Table S4): (1) initial plant size, (2) flowering onset, (3) size at flowering, (4) aboveground vegetative biomass, (5) reproductive biomass, (6) reproductive investment, (7) number of inflorescences, (8) LDMC and (9) osmotic potential. We used linear mixed-effects models for all analyses except for the number of inflorescences, for which we used a generalised linear mixed-effects model with Poisson error distribution. All models included temporal origin (ancestor vs. descendant), watering treatment (control vs. drought), herbivory treatment (control vs. damaged) and all possible interactions as fixed explanatory variables, as well as seed family and the spatial block within the greenhouse as random variables. In all models except for the analysis of early size we further included early size as a covariate. We analysed the generalized linear mixed-effects models for the number of inflorescences using model comparisons by stepwise adding the fixed factors and their interactions. Because of the large numbers of traits, species and model factors, we adjusted our *p*-values for the false discovery rate (FDR) following Benjamini and Hochberg (1995). All analyses were done in R (Version 4.0.2) using the packages *plyr* for data structuring (Wickham 2011), and *lme4* (Bates *et al.* 2015) and *lmerTest* (Kuznetsova 2017) for the analyses.

Calculation of Q_{ST} and F_{ST}

The comparison of quantitative genetic differentiation (Q_{ST}) with neutral molecular differentiation (F_{ST}) is a useful tool for understanding the relative importance of selective versus random

processes in trait differentiation (Merilä and Crnokrak 2001; McKay and Latta 2002). When comparing these indices, three outcomes are possible: $Q_{ST}>F_{ST}$ suggests that natural selection is the main cause of differentiation, $Q_{ST}\approx F_{ST}$ that genetic drift could be the sole driver of it (but contributions of drift and selection remain unclear), and $Q_{ST}<F_{ST}$ indicates the influence of stabilising selection (Leinonen et al. 2008). As all study species were outcrossing, and because we implemented a half-sibling experimental design using seed families, we used the approach of Petit et al. (2001) to calculate Q_{ST} for each trait except for osmotic potential where the small numbers of replicates did not permit this. Q_{ST} was estimated as $Q_{ST} = V_{POP} / (2 \times V_A + V_{POP}) = V_{POP} / (8 \times V_{FAM} + V_{POP})$, where V_{POP} is the phenotypic variance between the two temporal origins and V_A the genetic variance within temporal origins (Wright 1951). We calculated V_{POP} per trait by first running a linear model with early size, the single treatments and the treatment interaction as fixed factors (trait ~ early size + drought × herbivory). We then extracted the residuals and using these in a linear mixed-effects model including temporal origin, its treatment interactions (= V_{POP}) and seed families (= V_{FAM}) as random factors. We resampled data 999 times from the original dataset to estimate a mean value and bootstrapped standard error for the Q_{ST} of every measured trait.

We estimated neutral genetic differentiation (F_{ST}) between the two temporal origins based on 2257 to 5785 biallelic SNP markers per species, using the function *stamppFst* from the *R* package *StAMPP* (Pembleton *et al.* 2013). A more detailed description of the SNP genotyping can be found in Appendix p. 125–126. The raw data have been deposited in the Europoean Nucleotide Archive under the accession number PRJEB47887.

Results

Mean values of all measured traits are presented in Table S2 accompanied with reaction norm plots per species in Fig S1–S4. Detailed model results are shown in Table S3.

Differentiation between ancestral and contemporary plants

The frequency and strength of genetic differentiations between ancestors and descendants strongly differed among the studied traits. Genetic differentiation was particularly common in flowering onset, with significantly accelerated flowering in descendants of *Matthiola tricuspidata* by 3.5 days ($p = 0.006$) and *Clinopodium vulgare* by 8.5 days ($p = 0.017$), but the opposite change for *Leontodon hispidus* by 5.5 days ($p = 0.031$, Fig. 1B; Table 2). In two species, *M. tricuspidata*

(p = 0.03) and *C. vulgare* (p = 0.025), we found that the reproductive investment was significantly higher (*M. tricuspidata* + 3%, *C. vulgare* + 10%) in the descendant plants (Fig. 1G; Table 2). Ancestors and descendants of two species differed in their size at flowering, with larger descendants in *L. hispidus* (p = 0.03) but smaller ones in *C. vulgare* (p = 0.03, Fig. 1C; Table 2). For the remaining traits we only found differences in single species (Fig. 1; Table 2).

Past selection within the studied populations

Molecular marker differentiation based on SNP data ranged from F_{ST} = 0.005 (*L. hispidus*) to F_{ST} = 0.148 (*P. crassifolia*; Fig. 2). Our mixed-effects model results showed significant phenotypic differentiation between the two temporal origins in 12 out of 32 trait × species combinations (Table 2, except the osmotic potential). For 10 of these, Q_{ST} was higher than the corresponding F_{ST}, and only two showed a lower Q_{ST} (Fig. 2). The most consistent results were for the time of flowering onset and size at flowering, where Q_{ST} was higher than F_{ST} in three out of four species. In addition, the LDMC showed consistent results but the other way around. We found no differentiations for this trait between ancestors and descendants for *M. tricuspidata*, *P. crassifolia* and *C. vulgare* and also a lower Q_{ST} in comparison to the F_{ST} (Fig. 2A–C). Across all traits, phenotypic differentiation was strongest, and Q_{ST} always above F_{ST}, in *L. hispidus* (Fig. 2D), whereas in *M. tricuspidata* and *P. crassifolia* the Q_{ST} values were generally much lower, and in most cases below the estimated F_{ST} (Fig. 2A,B).

Treatment responses of ancestral vs. contemporary plants

The drought treatment strongly influenced plant traits in all four species (Table 2), whereas effects of simulated herbivory were much more moderate, with significant effects only on six traits in *M. tricuspidata* and on one trait in *P. crassifolia* (Table 2, Fig. S1 and S2). In ten cases, the responses of plant traits to our treatments depended on the temporal origin (treatment × origin interactions in Table 2), again mostly with regard to drought (9 out of 10 interactions) and in *M. tricuspidata* (6 out of ten interactions). For vegetative biomass and LDMC the observed interactions were respectively consistent across two species (*P. crassifolia*, *C. vulgare* and *M. tricuspidata, C. vulgare*), indicating that descendants decreased their vegetative biomass and their LDMC less under drought than their ancestors.

The overall most responsive and differentiated trait was the number of inflorescences in *M. tricuspidata,* with significant effects of both experimental treatments, and all possible two- and three-way interactions between drought, herbivory and temporal origin (Tables 2, Fig. 3B). For

example, descendants decreased the number of inflorescences more in response to drought and to herbivory, whereas the decrease in their ancestors was smaller (Fig. 3A).

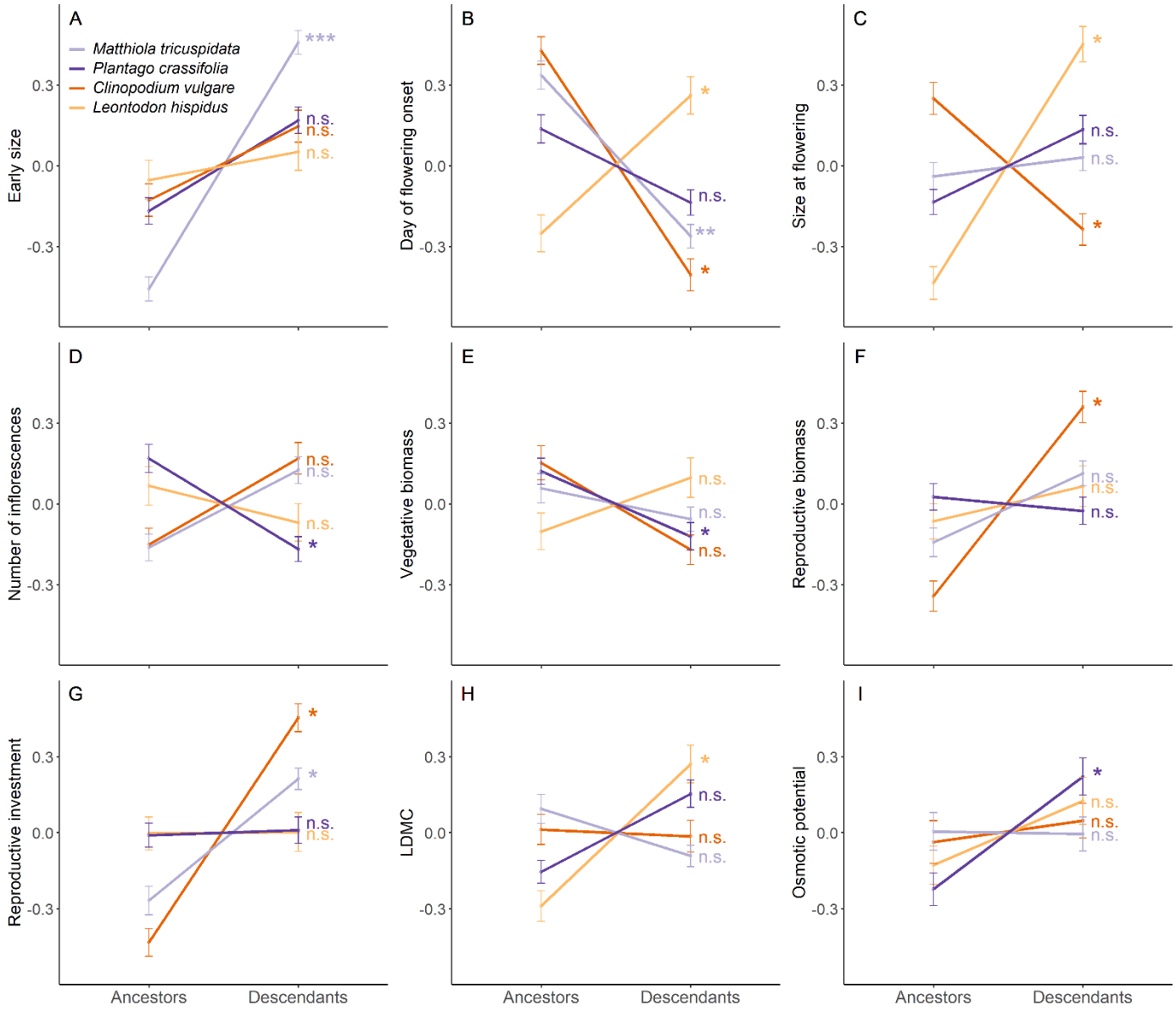

Figure 1 Changes from ancestors to descendants in the nine measured phenotypic traits for each of the four-individual species (coloured lines), with asterisks indicating significance levels of ancestor-descendant comparisons (* p<0.05, ** p<0.01, *** p<0.001, n.s. = not significant). All data are standardised; the error bars are standard errors.

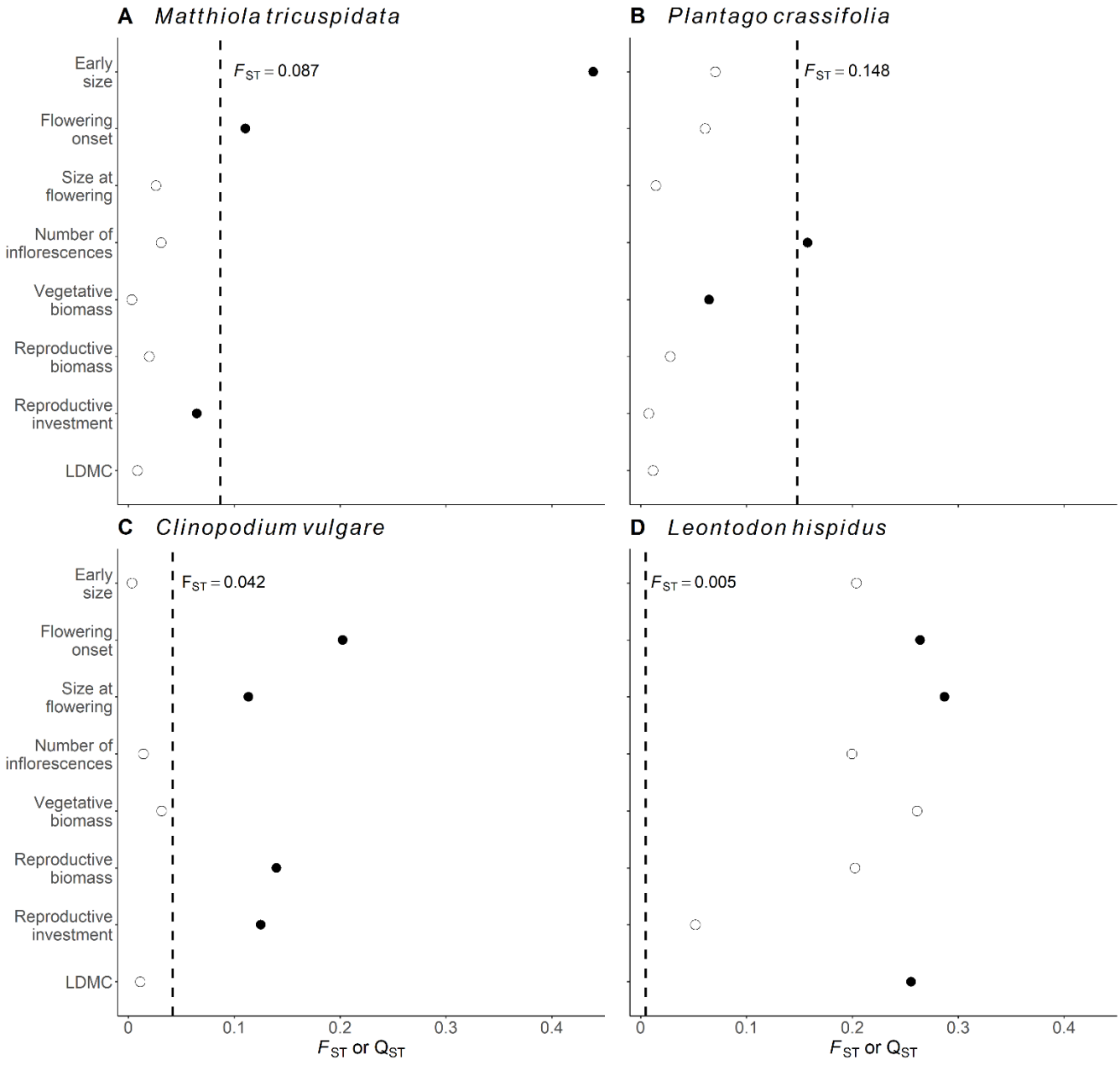

Figure 2 Q_{ST} values describing the phenotypic differentiation between ancestral and descendant plants (filled/empty circles), and how they compare to the respective F_{ST} values based on molecular data (dashed vertical lines), for each of the four studied species. Filled circles indicate traits with significant main effects for "origin" in the mixed models (Table 2). The standard errors of Q_{ST} values are too small for displaying.

Table 2 Results of statistical models testing the effects of temporal origin (ancestors vs. descendants), simulated drought and herbivory, and their interactions, on the growth, fitness and functional traits of *Matthiola tricuspidata* (Mt), *Plantago crassifolia* (Pc), *Clinopodium vulgare* (Cv) and *Leontodon hispidus* (Lh). Significant results are indicated by shading: light green $p<0.05$, green $p<0.01$, dark green $p<0.001$, with arrows indicating their directions: ↑ or ↓ if descendants have larger/smaller values in origin effect or the treatments (drought or herbivory) lead to larger/smaller values; ↗ or ↘ if descendants responded stronger to treatments than ancestors, and values increased/decreased compared to the control treatment; ↖ or ↙ if ancestors responded stronger to treatments than descendants, and values increased/decreased compared to the control; ↙/↗ is a special case where both temporal origins responded to the treatment, with decreasing values in the ancestors, but increasing values in descendants.

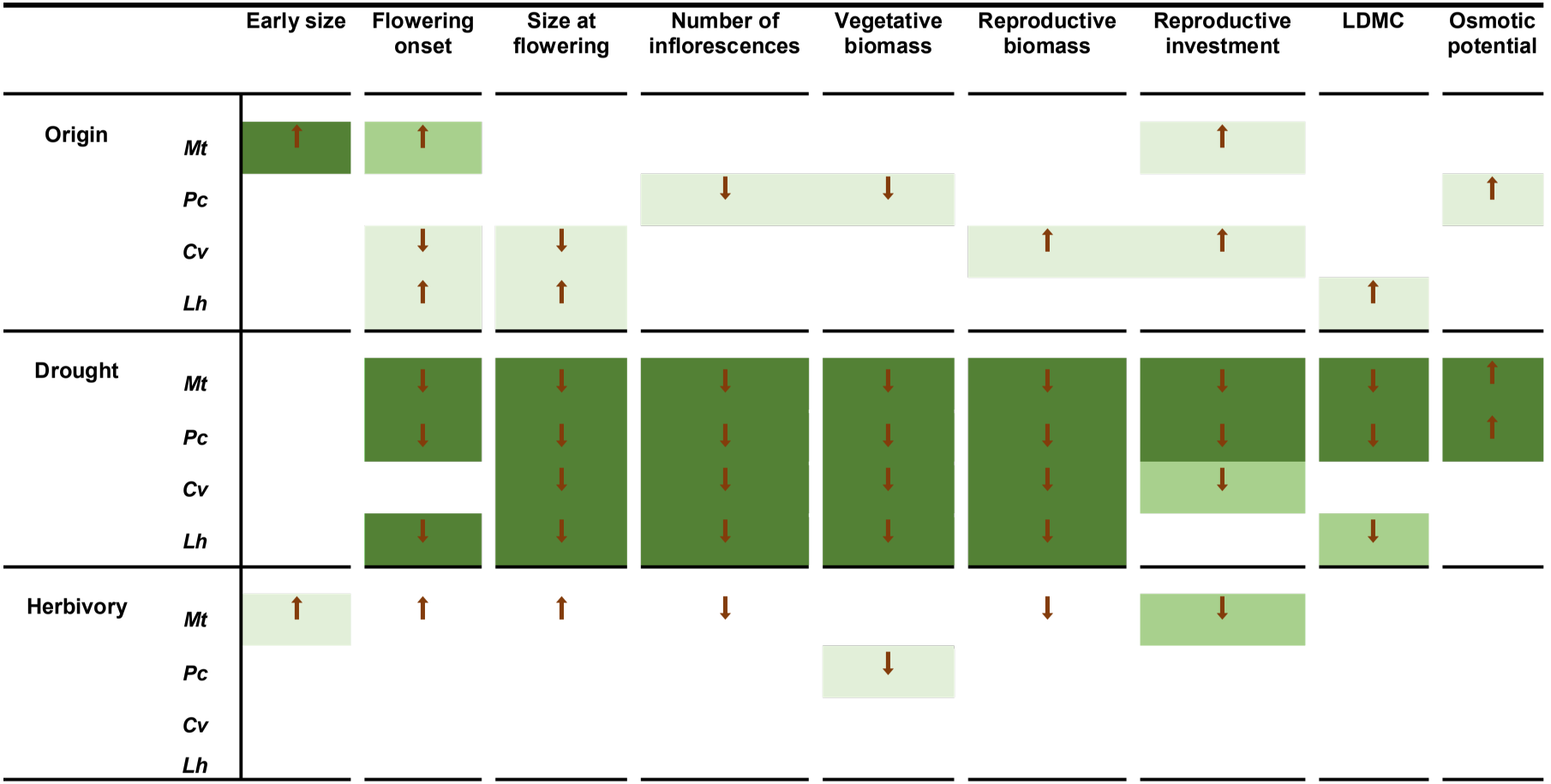

Table 2 continued

		Early size	Flowering onset	Size at flowering	Number of inflorescences	Vegetative biomass	Reproductive biomass	Reproductive investment	LDMC	Osmotic potential
Origin × Drought	*Mt*		↗	↘	↘			↙	↙	
	Pc					↙				↗
	Cv					↙			↙/↗	
	Lh									
Origin × Herbivory	*Mt*				↘					
	Pc									
	Cv									
	Lh									
Drought × Herbivory	*Mt*									
	Pc									
	Cv									
	Lh									
Origin × Drought × Herbivory	*Mt*									
	Pc									
	Cv									
	Lh									

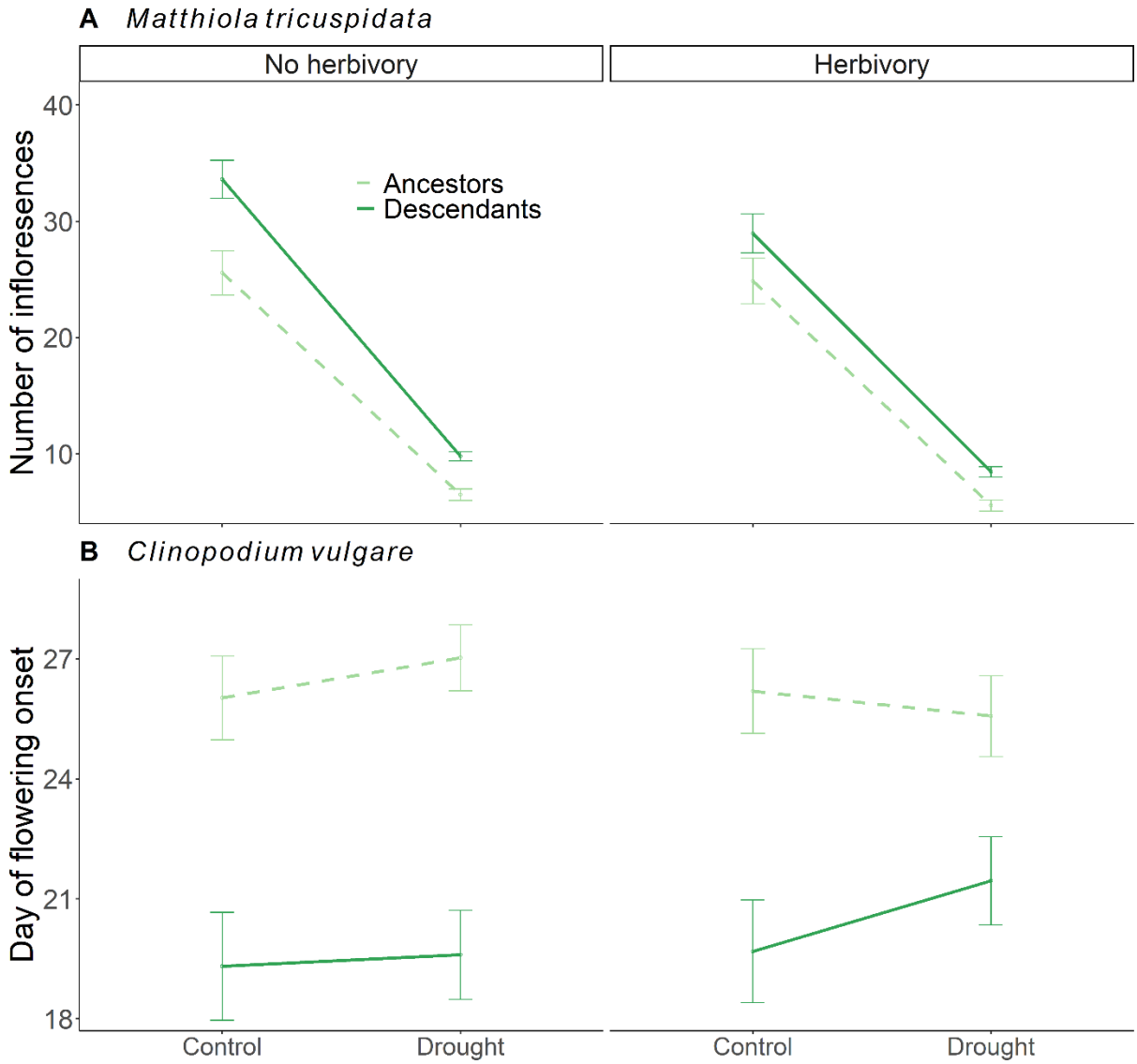

Figure 3 Significant three-way interactions between drought, herbivory and temporal origin for the number of inflorescences in *M. tricuspidata* (A) and day of flowering onset in *C. vulgare* (B). The data are mean values and their standard errors.

Discussion

Differentiation between ancestral and contemporary plants

The potential for rapid evolutionary changes not only depends on the strength of selection exerted by environmental changes, but also on the numbers of generations that have passed. All of our study species can reproduce within one year, so we assume that the studied plant populations underwent approximately the same number of sexual generations over the 21–26 years period since the ancestral seed were collected. We found that flowering onset was significantly advanced in the descendant compared to the ancestral plants in two species, and that there was a similar trend in a third species (*P. crassifolia*, $p = 0.07$). Accelerated reproduction is often considered an adaptation to increasingly drier and warmer conditions (Franks *et al.* 2007; Kigel *et al.* 2011; Metz *et al.* 2020), since early-flowering plants may have a better chance to escape summer droughts. Furthermore, climate change may also increase insect outbreaks (Bale *et al.* 2002) and foliar damage (Hamann *et al.* 2020) in the summer. Our findings therefore suggest a synergistic response to climate change and simultaneous herbivory as plants may escape from both stressors by shortening their life cycles (Pilson 2000; Kawagoe and Kudoh 2010). However, the phenotypic differentiation between ancestors and descendants depended strongly on the species. In contrast to the three other species, *L. hispidus* showed the opposite pattern, with descendants flowering later than their ancestors (Table 2, Fig. 1B). One explanation for this could be that this species originated from a site that was not managed in the 1990s. Since 2007, it has been grazed by sheep in the spring, and this alteration of management might have selected more strongly for later flowering (Völler *et al.* 2012) than climate changes selected in the opposite direction.

The faster development in terms of advanced flowering is also accompanied by faster early growth in the annual *M. tricuspidata*, with similar (non-significant) tendencies in the three other study species, possibly affecting the time when plant size thresholds for flowering are reached (Bolmgren and Cowan 2008; Sun and Frelich 2011). However, for the size at flowering results were inconsistent across species (Table 2, Fig. 1C), maybe reflecting differences in life-history strategies among the species, or differences in habitat conditions across the population origins. The descendants of the two Mediterranean species flowered earlier but did not differ in their size at flowering compared to their ancestors, whereas the descendants of *C. vulgare* flowered earlier and were smaller at the time of flowering. Therefore, it appears that the descendants of *M. tricuspidata* and *P. crassifolia* grew faster and thus reached size thresholds for flowering earlier (Sun and Frelich 2011), and that the descendants of *C. vulgare* evolved flowering onset at an earlier developmental stage. With both strategies life cycles are completed

faster, which is thought to benefit plants in disturbed and/or unpredictable environments (Grime 1977).

In summary, we find evolutionary changes towards accelerated life-cycles and increased reproduction or reproductive allocation in several species, e.g. higher reproductive biomass of descendants in *C. vulgare*, or a higher reproductive investment of descendants in *M. tricuspidata* and *C. vulgare*. The consistency of the changes across the studied species suggests that they are driven by adaptive evolutionary processes instead of random processes such as drift, most likely in response to increased environmental stress during summertime, especially summer droughts, within the last decades (Franks *et al.* 2007; Kigel *et al.* 2011; Metz *et al.* 2020).

Evolutionary processes

Comparisons of Q_{ST} (phenotypic differentiation) and F_{ST} (neutral molecular differentiation) can help to understand the importance of natural selection versus other, non-selective evolutionary processes such as genetic drift or gene flow as causes of population differentiation. We found F_{ST} values between 0.005 to 0.148 in our study species, with higher F_{ST} values in the two Mediterranean species, in particular *P. crassifolia*, but small values in the two temperate species *C. vulgare* and the self-incompatible *L. hispidus*. In contrast to the temperate species, the Mediterranean species come from frequently disturbed habitats, which might increase chances for non-adaptive, random processes through bottlenecks and/or immigration (Banks *et al.* 2013; Davies *et al.* 2016) and lead to stronger differentiation between ancestors and descendants. However, many other factors can influence molecular differentiation between populations, and its differences between species, such as mating system, pollination mode, seed dispersal (Gamba and Muchhala 2020), connectivity (Rousset 1997; Durka *et al.* 2017) and population size (van Treuren *et al.* 1991). Notwithstanding these uncertainties, our F_{ST} measurements are in the expected range. For example, Summers and colleagues resurrected seeds from the soil seed bank of the perennial *Schoenoplectus americanus*, ranging from 1900–1998, and collected new plant material from the same population in 2002 and found a maximum F_{ST} of 0.19 (Summers *et al.* 2018). Our F_{ST} results generally also support our study design and sampling strategy. With strong bottleneck events, or different sampling strategies we would probably have found much stronger molecular differentiation between ancestors and descendants (Lauterbach *et al.* 2011; Rucińska and Puchalski 2011).

The Q_{ST} of onset of flowering was larger than F_{ST} in three species (*M. tricuspidata*, *C. vulgare* and *L. hispidus*), suggesting directional selection as the most likely responsible evolutionary process (Leinonen *et al.* 2013). On the other hand, for *M. tricuspidata*, *P. crassifolia*

and *C. vulgare*, the Q_{ST} values of vegetative biomass and LDMC were smaller than F_{ST} values, indicating stabilizing selection on these traits. These observations are in line with other studies, which also found directional selection for flowering-related traits and stabilizing selection for vegetative traits (Chun *et al.* 2011; Kesselring *et al.* 2015), and it supports our idea above that the acceleration of life cycles is a key evolutionary adaptation in response to climate change, to escape from drought stress. Still, we should keep in mind that in our study we calculated Q_{ST} values for ancestral vs. descendant plants of a single population per species. The generality of our results is thus unclear and will require further testing across multiple populations. Another caveat is the small number of seed families per temporal origin for *L. hispidus*, which may have contributed to low F_{ST} and disproportionate high Q_{ST} estimates, as V_{FAM} was rather small, for this species, which questioned our conclusions of directional selection from Q_{ST}-F_{ST} comparisons.

Responses of ancestral vs. contemporary plants to drought and herbivory

The drought treatment generally had much stronger effects on the measured traits than the herbivory treatment, and there were also many more significant drought × origin interactions than herbivory × origin interactions. This could either be because herbivory was a weaker driver of natural selection in the studied populations during the last decades, or it could be because the simulated herbivory in our experiment was too weak to provoke stronger plant responses. This disparity of our two treatments in impacting the studied species could be the reason that we found hardly any evidence for trade-offs in adaptations to drought and herbivory (Sthultz *et al.* 2009; Pilson 2000; Nelson *et al.* 2017).

We generally observed the strongest patterns in *M. tricuspidata,* including the only significant herbivory main effects, most drought × origin interactions and the only significant herbivory × origin interaction. As *M. tricuspidata* was the only strictly annual species in our study it is possible that ancestors and descendants were stronger differentiated because of the higher effective number of generations available for evolutionary changes. We found for *M. tricuspidata* that descendants delayed flowering significantly less under drought than their ancestors did. This could be interpreted as evolution of stronger homeostasis under increasingly drier summers (Grene *et al.* 2011; de Kort *et al.* 2020), or as a more opportunistic phenology if water becomes available later in growing-season (Dyer *et al.* 2012). Besides differences in the plasticity of flowering time, we also found that the descendants decreased their number of inflorescences more strongly under both drought as well as herbivory conditions. Whether this represents evolution of a more opportunistic reproduction, or plants just suffered more under more stressful environmental conditions (Dyer *et al.* 2012; de Kort *et al.* 2020), cannot be answered by our

experiment, but the first explanation is supported by the weaker decreases of LDMC in *M. tricuspidata* descendants, a morphological trait that is positively related to plant resistance against drought and herbivory (Gardarin *et al.* 2014; Blumenthal *et al.* 2020). A similar pattern was found in *C. vulgare,* where descendants increased and ancestors decreased LDMC in response to drought. However, the unclear genetic background of the two temporal origins in *M. tricuspidata*, with large differences in the allelic richness, can serve as an alternative explanation for the numerous observed differentiations between ancestors and descendants in this species.

We found that the descendants of *P. crassifolia* increased their osmotic potential more strongly under drought than their ancestors did. The osmotic potential is directly related to the molar concentration of solutes in plant cells, which is tightly linked to the plant wilting point (Bartlett *et al.* 2012; Meinzer *et al.* 2016) and therefore drought tolerance (Kolb and Sperry 1999; Lenz *et al.* 2006; Májeková *et al.* 2019). At the same time, ancestors of *P. crassifolia* and also *C. vulgare* showed significantly greater decreases in biomass in response to drought. Our results thus strongly indicate that descendants of *P. crassifolia* have evolved greater plasticity in a functional trait that allows them to better cope with drought (Ackerly *et al.* 2000; Richards *et al.* 2006).

Conclusion

We studied four plant species from two biogeographic regions in Europe in a resurrection experiment, and we found evidence that the descendant populations of three species evolutionarily shortened their life cycles, presumably in response to climate change, during a period of only 21–26 years. Shortened life cycles may allow plants to escape increasingly frequent summer droughts and potential insect outbreaks. In our study the plants realized this through rapid seedling growth, earlier flowering onset and/or shifts in resource allocation. In addition to these evolutionary “escape strategies”, we also detected evolutionary changes in the osmotic potential in one species, and in LDMC in three species, which indicate evolution of greater drought and herbivory resistance through increased phenotypic plasticity in the descendant plants. Our quantitative genetic analysis indicated directional selection on several functional traits, supporting our hypothesis of adaptive evolutionary changes in the studied plant populations. Our study demonstrates the power of historical comparisons between banked seeds and current populations for studying rapid evolutionary changes. To gain deeper insights into evolutionary changes future studies should conduct transplantations of ancestors and descendants into their original habitat and include longer-term fitness measures.

Acknowledgments

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Supplementary material

Fig. S1 Mean values (sometimes transformed) and standard errors for all measured traits in *Matthiola tricuspidata*. The results are separated by treatments (watering treatment on the x-axis) and the temporal origins.

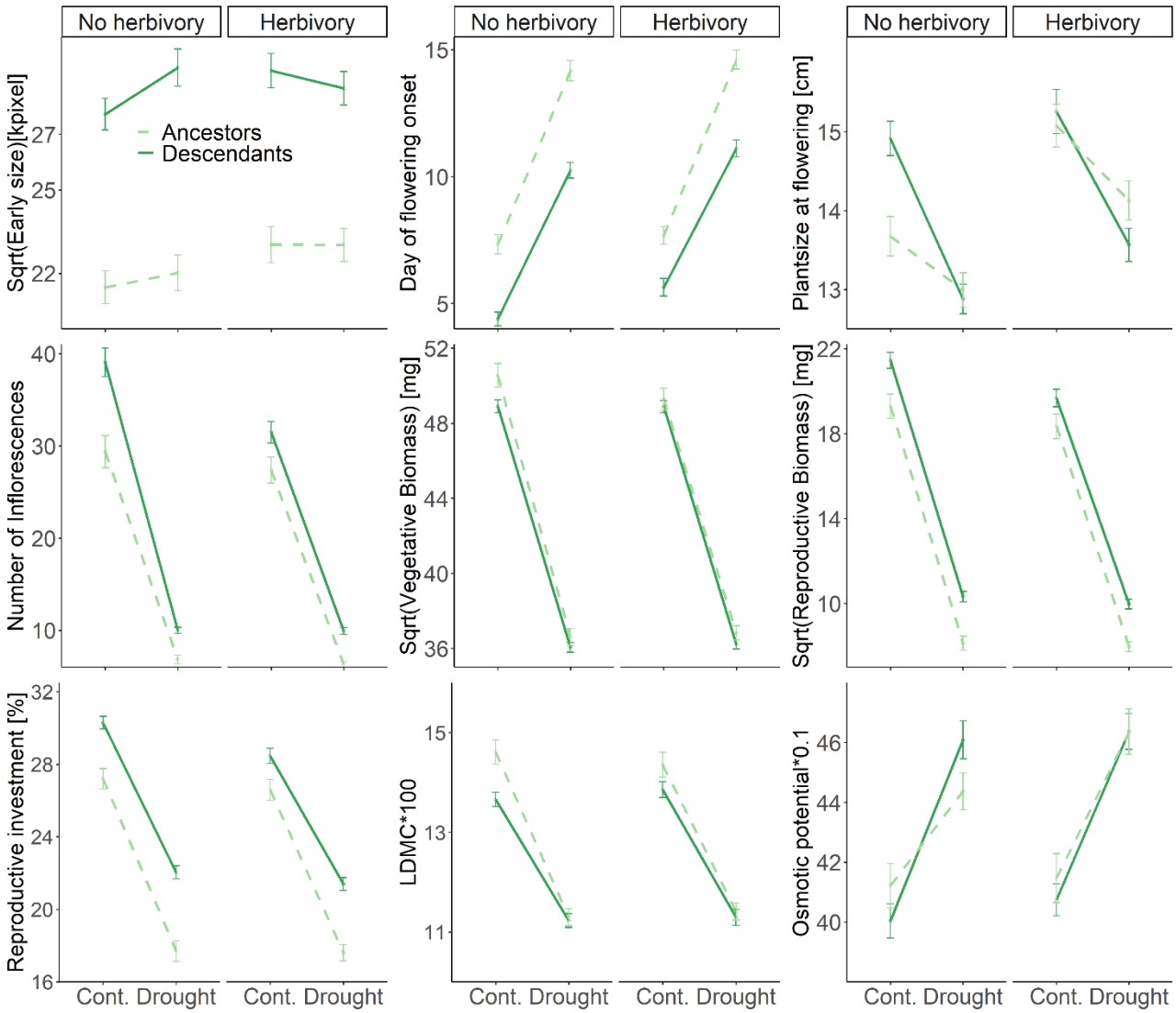

Fig. S2 Mean values (sometimes transformed) and standard errors for all measured traits in *Plantago crassifolia*. The results are separated by treatments (watering treatment on the x-axis) and the temporal origins.

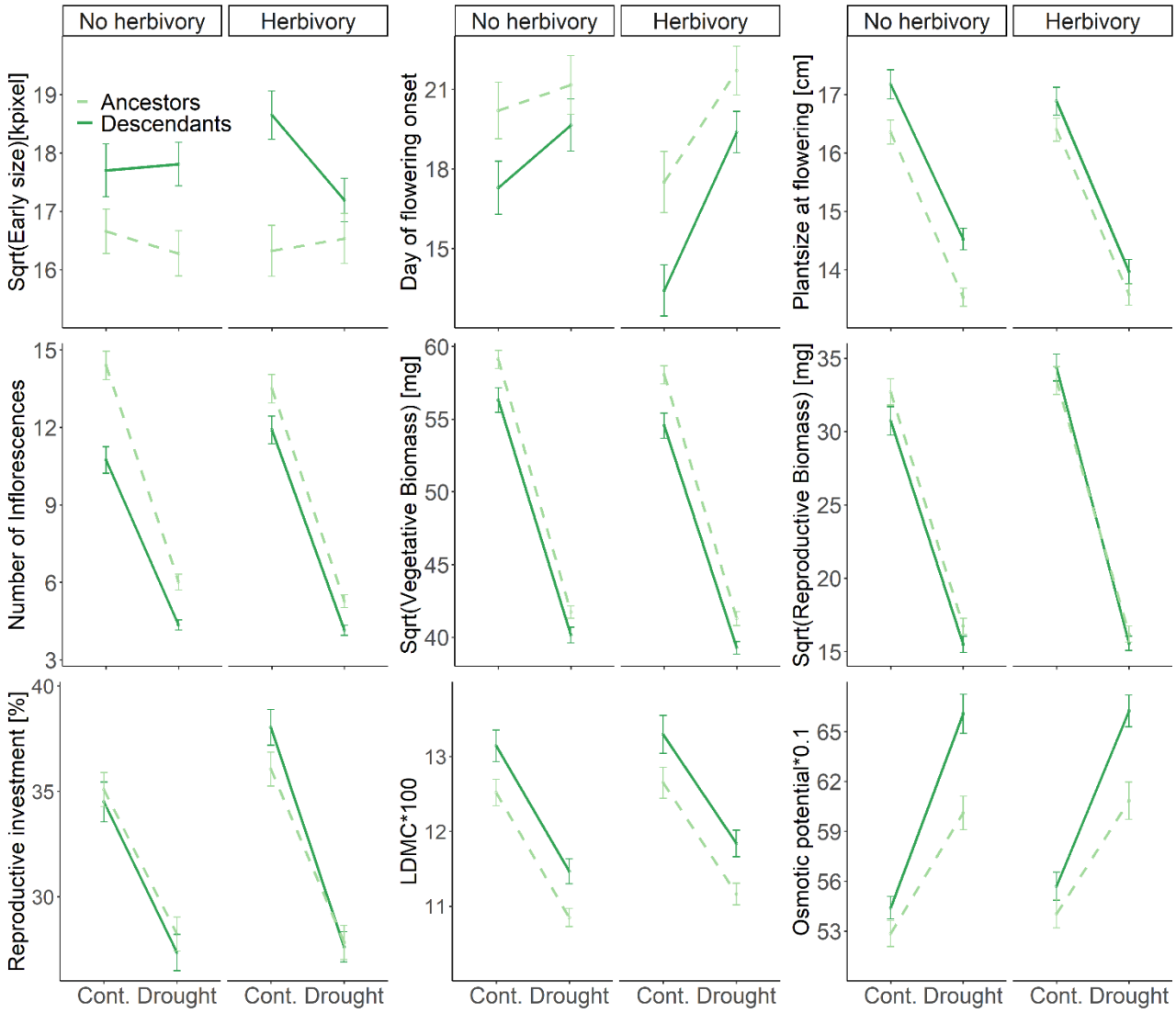

Fig. S3 Mean values (sometimes transformed) and standard errors for all measured traits in *Clinopodium vulgare*. The results are separated by treatments (watering treatment on the x-axis) and the temporal origins.

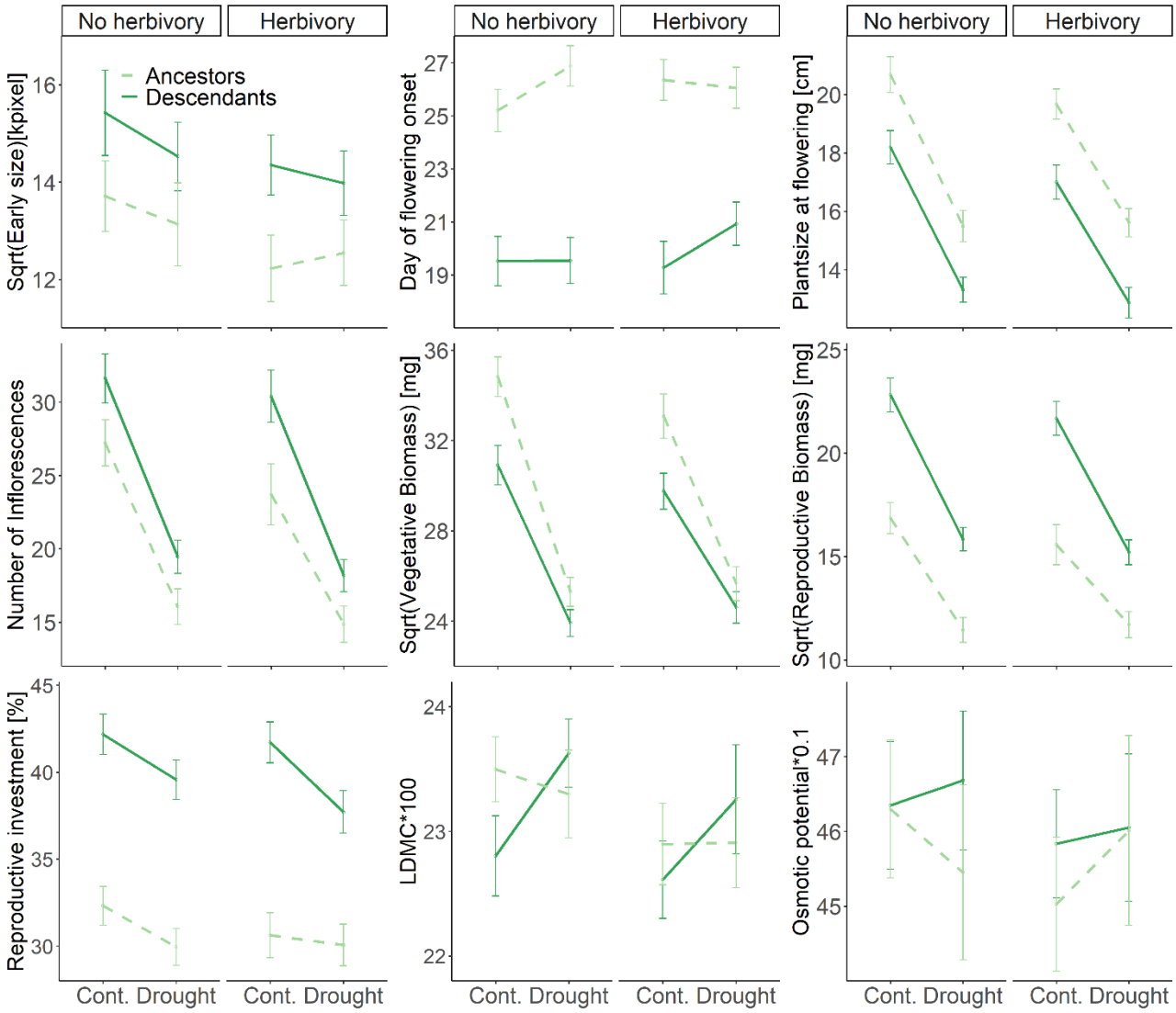

Fig. S4 Mean values (sometimes transformed) and standard errors for all measured traits in *Leontodon hispidus*. The results are separated by treatments (watering treatment on the x-axis) and the temporal origins.

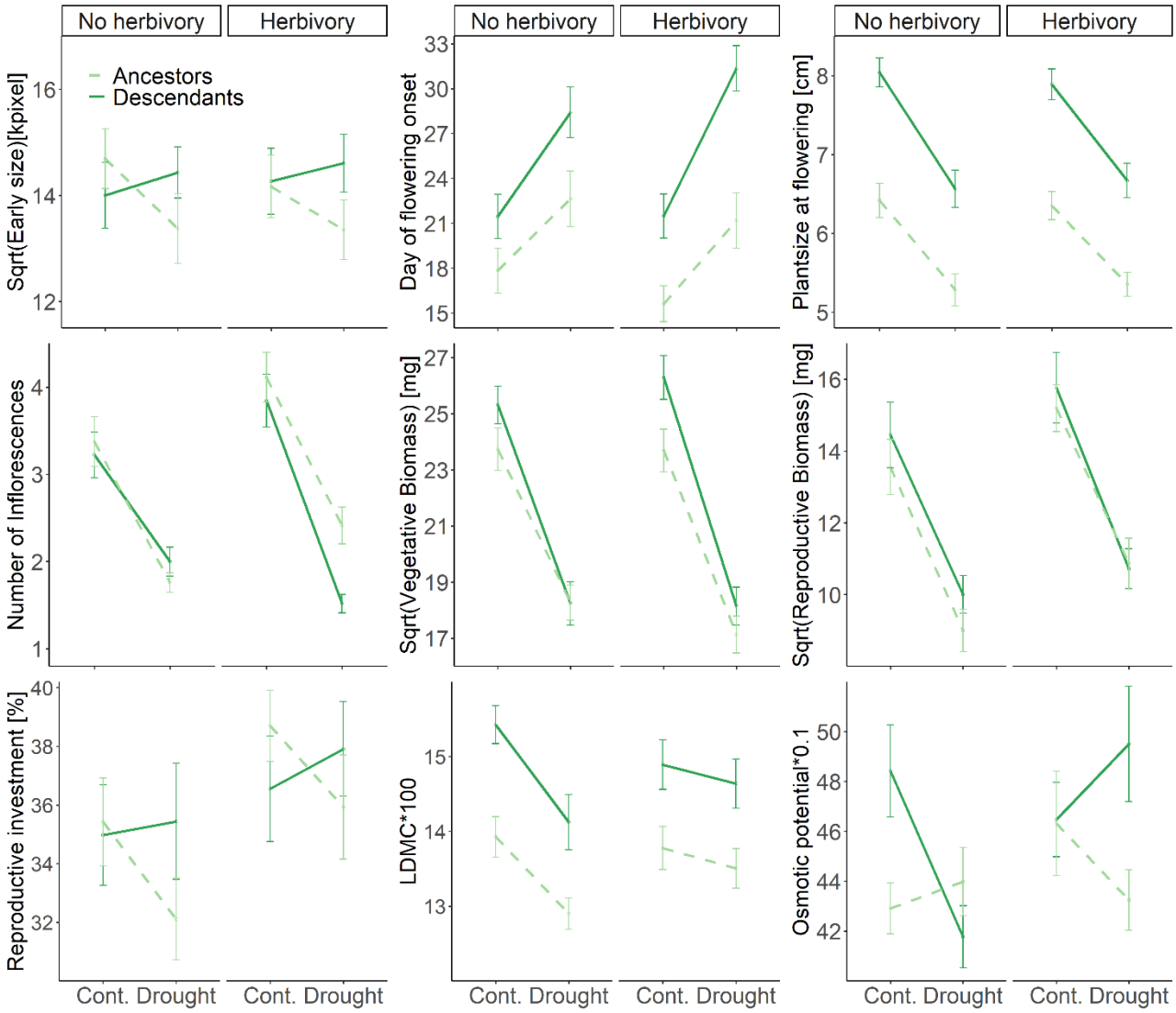

Table S1 Study species used in the experiment with details on plant family, ancestor collection year, amount of stored seeds in the seedbanks, number of mother plants sampled in 2018, estimated population size in 2018, the number sown and

Species	Family	Ancestor collection year	Amount of stored seeds	Number of mother plants sampled in 2018	Estimated population size in 2018	Number of sown seeds / germination rate	
						Ancestors	Descendants
Matthiola tricuspidata	Brassicaceae	1994	1000	15	30	100 / 95%	100 / 98%
Plantago crassifolia	Plantaginaceae	1994	800	10	15	100 / 29%	100 / 29%
Clinopodium vulgare	Lamiaceae	1992	1000	47	500	200 / 75%	200 / 90%
Leontodon hispidus	Asteraceae	1995	1000	20	100	300 / 30%	300 / 80%

Table S2 Mean values of the measured traits including the standard error separated by the temporal origins (ancestors and descendants) and the four different treatments

Treatment	Temporal origin	Early size [kpixel]	Flowering onset	Size at flowering [cm]	Number of inflorescences	Vegetative biomass [mg]	Reproductive biomass [mg]	Reproductive investment	LDMC	Osmotic potential
Matthiola tricuspidata										
Control	Anc.	496 +/- 28	7.3 +/- .4	14 +/- .3	29.4 +/- 1.7	2594 +/- 62	404 +/- 21	.13 +/- .006	.15 +/- .003	412 +/- 7
	Des.	801 +/- 32	4.4 +/- .3	15 +/- .2	39.0 +/- 1.6	2403 +/- 33	474 +/- 16	.16 +/- .004	.14 +/- .001	400 +/- 6
Drought	Anc.	526 +/- 30	14.2 +/-.4	13+/- .2	6.8 +/- .5	1359 +/- 33	77 +/- 7	.05 +/- .004	.11 +/- .002	443 +/- 6
	Des.	908 +/- 42	13 +/-.3	13 +/- .2	1.0 +/- .3	1306 +/- 18	113 +/- 6	.08 +/- .003	.11+/- .001	460 +/- 6
Herbivory	Anc.	573 +/- 31	7.7 +/- .4	15 +/- .4	27.4 +/- 1.4	2463 +/- 58	370 +/- 20	.13 +/- .005	.14 +/- .003	414 +/- 8
	Des.	896 +/- 37	5.6 +/- .4	15 +/- .3	31.5 +/- 1.2	2400 +/- 31	404 +/- 16	.14+/- .005	.14 +/- .002	407 +/- 5
Drought + Herbivory	Anc.	565 +/- 30	14.6 +/-.4	14 +/- .3	6.3 +/- .4	1371 +/- 28	69 +/- 4	.05 +/- .003	.11 +/- .002	463 +/- 8
	Des.	858 +/- 35	11.1 +/-.3	14 +/- .2	9.9+/- .4	1316 +/- 17	105 +/- 5	.07 +/- .003	.11 +/- .002	463 +/- 6
Plantago crassifolia										
Control	Anc.	292 +/- 13	20 +/- 1.1	16 +/- .2	14.4 +/- .6	3531 +/- 72	1147 +/- 50	.24 +/- .01	.13 +/- .002	528 +/- 8
	Des.	334 +/- 16	17 +/- 1.0	17 +/- .3	1.7 +/- .5	3242 +/- 95	1037 +/- 51	.24 +/- .012	.13 +/- .002	544 +/- 7
Drought	Anc.	280 +/- 13	21 +/- 1.1	14 +/- .2	6.0 +/- .3	1760 +/-36	308 +/- 17	.15 +/- .008	.11 +/- .001	601 +/- 10
	Des.	331 +/- 13	20 +/- 1.0	15 +/- .2	4.3 +/- .2	1641 +/-42	270 +/- 16	.14 +/- .009	.11+/- .002	660 +/- 12
Herbivory	Anc.	285 +/- 15	18 +/- 1.2	16 +/- .2	13.5 +/- .6	3406 +/- 73	1208 +/- 55	.26 +/- .011	.13 +/- .002	540 +/- 9
	Des.	365 +/- 16	13 +/- 1.0	17 +/- .2	11.9 +/- .5	3048 +/- 89	1263 +/- 54	.29 +/- .013	.13 +/- .003	556 +/- 9
Drought + Herbivory	Anc.	291 +/- 15	22 +/- .9	14 +/- .2	5.3 +/- .3	1727 +/- 42	292 +/- 16	.15 +/- .008	.11 +/- .001	608 +/- 11
	Des.	309 +/- 13	19 +/- .8	14 +/- .2	4.1 +/- .2	1561 +/- 34	266 +/- 14	.14 +/- .007	.12 +/- .002	662 +/- 10
Clinopodium vulgare										
Control	Anc.	225 +/- 22	25 +/- .8	21 +/- .6	27 +/- 1.6	1268 +/- 67	324 +/- 26	.21 +/- .014	.23 +/- .003	462 +/- 9
	Des.	286 +/- 32	20 +/- .9	18 +/- .6	32 +/- 1.7	1003 +/- 55	562 +/- 35	.36 +/- .019	.23 +/- .003	463 +/- 9
Drought	Anc.	224 +/- 29	27 +/- .8	15 +/- .5	16 +/- 1.2	669 +/- 34	157 +/- 15	.18 +/- .013	.23 +/- .004	454 +/- 12
	Des.	242 +/- 24	20 +/- .9	13 +/- .4	19 +/- 1.1	594 +/- 28	271 +/- 18	.32 +/- .019	.24 +/- .003	466 +/- 8
Herbivory	Anc.	183 +/- 19	26 +/- .8	20 +/- .5	24+/- 2.1	1163 +/- 67	309 +/- 33	.19 +/- .015	.23 +/- .004	450 +/- 9
	Des.	230 +/- 19	19 +/- 1	17 +/- .6	30 +/- 1.8	925 +/- 49	511 +/- 34	.36 +/- .019	.23 +/- .004	458 +/- 7
Drought + Herbivory	Anc.	190 +/- 20	26 +/- .8	16 +/- .5	15 +/- 1.2	699 +/- 40	166 +/- 16	.18 +/- .015	.23 +/- .003	460 +/- 13
	Des.	223 +/-22	21 +/- .8	13 +/- .5	18 +/- 1.1	635 +/- 34	254 +/- 17	.29 +/- .018	.23+/- .003	460 +/- 10
Leontodon hispidus										
Control	Anc.	231 +/- 18	18 +/- 1.5	6.4 +/- .2	3.4 +/- .3	590 +/- 37	212 +/- 23	.26 +/- .023	.14 +/- .003	429 +/- 10
	Des.	215 +/- 18	21 +/- 1.5	8.0 +/- .2	3.2 +/- .3	663 +/- 36	250 +/- 28	.26 +/- .025	.15 +/- .003	484 +/- 18
Drought	Anc.	200 +/- 19	23 +/- 1.9	5.3 +/- .2	1.8 +/- .1	352 +/- 24	97 +/- 14	.21 +/- .02	.13 +/- .002	439 +/- 14
	Des.	220 +/- 14	28 +/- 1.7	6.6 +/- .2	2.0 +/- .2	362 +/- 29	113 +/- 11	.26 +/- .031	.14 +/- .004	417 +/- 12
Herbivory	Anc.	217 +/- 18	16 +/- 1.2	6.4 +/- .2	4.1 +/-.3	589 +/- 37	251 +/- 20	.30 +/- .019	.14 +/- .003	463 +/- 21
	Des.	222 +/- 19	21 +/- 1.5	7.9 +/- .2	3.8 +/- .3	721 +/- 41	296 +/- 33	.28 +/- .027	.15 +/- .003	465 +/- 15
Drought + Herbivory	Anc.	193 +/- 16	21 +/- 1.8	5.4 +/- .2	2.4 +/- .2	314 +/- 23	142 +/- 18	.27 +/- .027	.14 +/- .003	432 +/- 12
	Des.	228 +/- 16	31 +/- 1.5	6.7 +/- .2	1.5 +/- .1	351 +/- 26	130 +/- 13	.30 +/- .026	.15 +/- .003	495 +/- 23

Table S3 Results from statistical models testing the effects of temporal origin (ancestors vs. descendants), simulated drought and herbivory, and their interactions, on the growth, fitness and functional traits of *Matthiola tricuspidata* (Mt), *Plantago crassifolia* (Pc), *Clinopodium vulgare* (Cv) and *Leontodon hispidus* (Lh) Significant results ($p<0.05$) are written in bold and are indicated by shading: light green $p<0.05$, green $p<0.01$, dark green $p<0.001$

		Early size	Flowering onset	Size at flowering	Number of inflorescences	Vegetative biomass	Reproductive biomass	Reproductive investment	LDMC	Osmotic potential
Origin	***Mt***	**$F_{1,18.0} = 47.0$ $p < 0.001$**	**$F_{1,18.2} = 14.3$ $p = 0.006$**	$F_{1,18.7} = 0.4$ $p = 0.622$	$Chi^2_1 = 4.7$ $p = 0.667$	$F_{1,18.6} = 1.9$ $p = 0.269$	$F_{1,17.8} = 3.1$ $p = 0.171$	**$F_{1,17.3} = 8.3$ $p = 0.030$**	$F_{1,19.0} = 1.4$ $p = 0.325$	$F_{1,19.6} = 0.0$ $p = 0.881$
	Pc	$F_{1,18.1} = 5.8$ $p = 0.051$	$F_{1,18.0} = 5.0$ $p = 0.067$	$F_{1,17.9} = 1.3$ $p = 0.345$	**$Chi^2_1 = 7.6$ $p = 0.0$**	**$F_{1,18.2} = 6.2$ $p = 0.049$**	$F_{1,18.2} = 0.9$ $p = 0.403$	$F_{1,18.1} = 0.1$ $p = 0.793$	$F_{1,18.0} = 2.5$ $p = 0.192$	**$F_{1,17.7} = 9.2$ $p = 0.032$**
	Cv	$F_{1,13.9} = 1.0$ $p = 0.419$	**$F_{1,14.1} = 14.6$ $p = 0.017$**	**$F_{1,13.9} = 8.0$ $p = 0.030$**	$Chi^2_1 = 2.1$ $p = 0.227$	$F_{1,14.1} = 3.0$ $p = 0.188$	**$F_{1,14.1} = 10.2$ $p = 0.025$**	**$F_{1,14.0} = 9.4$ $p = 0.025$**	$F_{1,13.9} = 0.3$ $p = 0.656$	$F_{1,13.9} = 0.0$ $p = 0.853$
	Lh	$F_{1,387.1} = 1.1$ $p = 0.370$	**$F_{1,8.3} = 11.0$ $p = 0.031$**	**$F_{1,8.2} = 16.1$ $p = 0.030$**	$Chi^2_1 = 1.2$ $p = 0.370$	$F_{1,8.1} = 1.7$ $p = 0.370$	$F_{1,278.0} = 0.8$ $p = 0.408$	$F_{1,9.3} = 0.1$ $p = 0.773$	**$F_{1,8.1} = 13.1$ $p = 0.030$**	$F_{1,8.1} = 2.0$ $p = 0.370$
Drought	***Mt***	$F_{1,762.5} = 2.1$ $p = 0.149$	**$F_{1,657.7} = 883.0$ $p < 0.001$**	**$F_{1,659.2} = 61.8$ $p < 0.001$**	**$Chi^2_1 = 5240$ $p < 0.001$**	**$F_{1,743.0} = 2836.6$ $p < 0.001$**	**$F_{1,663.8} = 1893.7$ $p < 0.001$**	**$F_{1,664.4} = 868.9$ $p < 0.001$**	**$F_{1,734.7} = 647.5$ $p < 0.001$**	**$F_{1,365.7} = 163.3$ $p < 0.001$**
	Pc	$F_{1,763.6} = 2.0$ $p = 0.154$	**$F_{1,665.0} = 32.1$ $p < 0.001$**	**$F_{1,664.4} = 419.5$ $p < 0.001$**	**$Chi^2_1 = 1304.5$ $p < 0.001$**	**$F_{1,759.6} = 1860.0$ $p < 0.001$**	**$F_{1,703.5} = 1374.6$ $p < 0.001$**	**$F_{1,702.7} = 288.3$ $p < 0.001$**	**$F_{1,756.8} = 179.3$ $p < 0.001$**	**$F_{1,366.8} = 223.9$ $p < 0.001$**
	Cv	$F_{1,518.7} = 0.8$ $p = 0.429$	$F_{1,449.9} = 3.1$ $p = 0.116$	**$F_{1,451.4} = 146.7$ $p < 0.001$**	**$Chi^2_1 = 660.7$ $p < 0.001$**	**$F_{1,508.5} = 232.0$ $p < 0.001$**	**$F_{1,486.9} = 188.3$ $p < 0.001$**	**$F_{1,487.2} = 10.4$ $p = 0.002$**	$F_{1,506.5} = 2.8$ $p = 0.123$	$F_{1,292.6} = 0.3$ $p = 0.583$
	Lh	$F_{1,385.2} = 0.6$ $p = 0.512$	**$F_{1,268.5} = 28.6$ $p < 0.001$**	**$F_{1,264.2} = 92.8$ $p < 0.001$**	**$Chi^2_1 = 86.7$ $p < 0.001$**	**$F_{1,347.4} = 232.7$ $p < 0.001$**	**$F_{1,278.0} = 49.7$ $p < 0.001$**	$F_{1,258.5} = 0.4$ $p = 0.524$	**$F_{1,348.4} = 10.7$ $p = 0.002$**	$F_{1,255.8} = 1.1$ $p = 0.367$
Herbivory	***Mt***	**$F_{1,762.7} = 4.7$ $p = 0.047$**	**$F_{1,657.5} = 14.1$ $p < 0.001$**	**$F_{1,658.8} = 25.2$ $p <0.001$**	**$Chi^2_1 = 68.0$ $p <0.001$**	$F_{1,743.4} = 1.7$ $p = 0.221$	**$F_{1,663.7} = 13.4$ $p < 0.001$**	**$F_{1,664.4} = 9.2$ $p = 0.005$**	$F_{1,735.8} = 0.0$ $p = 0.874$	$F_{1,368.6} = 3.9$ $p = 0.063$
	Pc	$F_{1,762.8} = 0.1$ $p = 0.719$	$F_{1,661.7} = 3.5$ $p = 0.095$	$F_{1,661.1} = 2.4$ $p = 0.152$	$Chi^2_1 = 4.7$ $p = 0.095$	**$F_{1,757.1} = 8.8$ $p = 0.028$**	$F_{1,701.8} = 3.6$ $p = 0.095$	$F_{1,701.3} = 4.2$ $p = 0.095$	$F_{1,754.0} = 4.0$ $p = 0.095$	$F_{1,369.6} = 1.9$ $p = 0.184$
	Cv	$F_{1,518.2} = 3.9$ $p = 0.148$	$F_{1451.4} = 0.02$ $p = 0.885$	$F_{1,451.3} = 3.9$ $p = 0.148$	$Chi^2_1 = 5.9$ $p = 0.140$	$F_{1,507.1} = 0.4$ $p = 0.602$	$F_{1,486.3} = 1.0$ $p = 0.488$	$F_{1,487.5} = 1.1$ $p = 0.488$	$F_{1,505.9} = 1.2$ $p = 0.488$	$F_{1,295.0} = 0.4$ $p = 0.602$
	Lh	$F_{1,388.0} = 0.0$ $p = 0.953$	$F_{1,266.5} = 0.1$ $p = 0.953$	$F_{1,263.0} = 0.0$ $p = 0.953$	$Chi^2_1 = 2.3$ $p = 0.444$	$F_{1,347.9} = 0.1$ $p = 0.953$	$F_{1,278.0} = 4.2$ $p = 0.281$	$F_{1,256.8} = 3.5$ $p = 0.281$	$F_{1,349.5} = 0.0$ $p = 0.953$	$F_{1,256.0} = 1.7$ $p = 0.444$

Table S3 continued

		Early size	Flowering onset	Size at flowering	Number of inflorescences	Vegetative biomass	Reproductive biomass	Reproductive investment	LDMC	Osmotic potential
Origin × Drought	***Mt***	$F_{1,762.6} = 0.1$ $p = 0.754$	**$F_{1,658.0} = 11.5$**	**$F_{1,659.4} = 9.7$ $p = 0.004$**	**$Chi^2_1 = 35.3$ $p < 0.001$**	$F_{1,743.0} = 0.7$ $p = 0.453$	$F_{1,664.3} = 1.9$ $p = 0.220$	**$F_{1,665.1} = 10.0$ $p = 0.004$**	**$F_{1,735.0} = 6.0$ $p = 0.026$**	$F_{1,369.4} = 4.3$ $p = 0.059$
	Pc	$F_{1,762.3} = 2.3$ $p = 0.193$	$F_{1,661.2} = 2.4$ $p = 0.193$	$F_{1,660.5} = 0.1$ $p = 0.924$	$Chi^2_1 = 4.5$ $p = 0.100$	**$F_{1,755.7} = 5.5$ $p = 0.048$**	$F_{1,703.0} = 1.1$ $p = 0.368$	$F_{1,702.0} = 3.3$ $p = 0.156$	$F_{1,751.4} = 0.0$ $p = 0.934$	**$F_{1,366.4} = 11.5$ $p = 0.007$**
	Cv	$F_{1,516.2} = 0.4$ $p = 0.681$	$F_{1,450.9} = 0.2$ $p = 0.735$	$F_{1,448.5} = 0.0$ $p = 0.991$	$Chi^2_1 = 1.7$ $p = 0.358$	**$F_{1,506.2} = 7.0$ $p = 0.049$**	$F_{1,484.5} = 5.8$ $p = 0.062$	$F_{1,487.1} = 2.0$ $p = 0.355$	**$F_{1,505.1} = 6.4$ $p = 0.049$**	$F_{1,294.8} = 0.5$ $p = 0.681$
	Lh	$F_{1,385.3} = 3.2$ $p = 0.462$	$F_{1,267.7} = 1.6$ $p = 0.481$	$F_{1,263.2} = 0.9$ $p = 0.506$	$Chi^2_1 = 2.8$ $p = 0.506$	$F_{1,347.7} = 2.7$ $p = 0.462$	$F_{1,278.0} = 0.0$ $p = 0.972$	$F_{1,255.4} = 1.7$ $p = 0.481$	$F_{1,348.1} = 0.0$ $p = 0.972$	$F_{1,255.5} = 0.0$ $p = 0.972$
Origin × Herbivory	***Mt***	$F_{1,762.3} = 0.3$ $p = 0.395$	$F_{1,656.1} = 1.0$ $p = 0.395$	$F_{1,657.5} = 4.5$ $p = 0.090$	**$Chi^2_1 = 9.4$ $p = 0.020$**	$F_{1,742.0} = 1.2$ $p = 0.395$	$F_{1,661.8} = 0.4$ $p = 0.542$	$F_{1,661.8} = 1.1$ $p = 0.395$	$F_{1,733.3} = 0.9$ $p = 0.395$	$F_{1,367.4} = 1.1$ $p = 0.395$
	Pc	$F_{1,763.3} = 0.5$ $p = 0.714$	$F_{1,663.3} = 0.1$ $p = 0.714$	$F_{1,662.8} = 2.6$ $p = 0.482$	$Chi^2_1 = 4.1$ $p = 0.391$	$F_{1,758.6} = 0.9$ $p = 0.714$	$F_{1,702.1} = 0.3$ $p = 0.714$	$F_{1,701.7} = 0.3$ $p = 0.714$	$F_{1,755.7} = 0.3$ $p = 0.714$	$F_{1,371.4} = 0.2$ $p = 0.714$
	Cv	$F_{1,518.4} = 0.1$ $p = 0.924$	$F_{1,451.2} = 0.4$ $p = 0.924$	$F_{1,451.8} = 0.2$ $p = 0.924$	$Chi^2_1 = 1.4$ $p = 0.924$	$F_{1,508.5} = 0.2$ $p = 0.924$	$F_{1,487.6} = 0.3$ $p = 0.924$	$F_{1,487.3} = 0.1$ $p = 0.924$	$F_{1,506.7} = 0.1$ $p = 0.924$	$F_{1,297.0} = 0.0$ $p = 0.950$
	Lh	$F_{1,385.9} = 0.3$ $p = 0.720$	$F_{1,265.2} = 1.3$ $p = 0.720$	$F_{1,262.7} = 0.0$ $p = 0.891$	$Chi^2_1 = 0.5$ $p = 0.720$	$F_{1,347.2} = 1.7$ $p = 0.720$	$F_{1,278.0} = 0.3$ $p = 0.720$	$F_{1,253.4} = 0.2$ $p = 0.720$	$F_{1,347.0} = 0.3$ $p = 0.720$	$F_{1,254.9} = 0.6$ $p = 0.720$
Drought × Herbivory	***Mt***	$F_{1,763.2} = 2.8$ $p = 0.210$	$F_{1,657.5} = 0.2$ $p = 0.725$	$F_{1,658.7} = 0.1$ $p = 0.725$	**$Chi^2_1 = 8.9$ $p = 0.023$**	$F_{1,743.5} = 3.4$ $p = 0.195$	$F_{1,663.9} = 4.4$ $p = 0.166$	$F_{1,664.6} = 1.0$ $p = 0.334$	$F_{1,736.2} = 0.3$ $p = 0.730$	$F_{1,368.8} = 0.3$ $p = 0.730$
	Pc	$F_{1,762.6} = 0.0$ $p = 0.900$	$F_{1,662.4} = 3.7$ $p = 0.315$	$F_{1,661.6} = 0.2$ $p = 0.723$	$Chi^2_1 = 0.5$ $p = 0.717$	$F_{1,756.3} = 2.0$ $p = 0.315$	$F_{1,701.6} = 2.2$ $p = 0.315$	$F_{1,701.2} = 2.1$ $p = 0.315$	$F_{1,752.8} = 1.6$ $p = 0.315$	$F_{1,364.8} = 0.4$ $p = 0.321$
	Cv	$F_{1,515.6} = 0.4$ $p = 0.970$	$F_{1,450.8} = 0.8$ $p = 0.970$	$F_{1,447.9} = 0.2$ $p = 0.970$	$Chi^2_1 = 0.7$ $p = 0.970$	$F_{1,505.2} = 2.9$ $p = 0.657$	$F_{1,484.7} = 0.6$ $p = 0.970$	$F_{1,487.2} = 0.0$ $p = 0.970$	$F_{1,504.6} = 0.0$ $p = 0.970$	$F_{1,293.6} = 0.2$ $p = 0.970$
	Lh	$F_{1,386.0} = 0.1$ $p = 0.911$	$F_{1,267.7} = 0.1$ $p = 0.911$	$F_{1,263.5} = 4.0$ $p = 0.208$	$Chi^2_1 = 0.9$ $p = 0.911$	$F_{1,347.6} = 0.0$ $p = 0.911$	$F_{1,278.0} = 0.0$ $p = 0.911$	$F_{1,256.6} = 0.0$ $p = 0.911$	$F_{1,348.6} = 4.6$ $p = 0.208$	$F_{1,256.7} = 1.1$ $p = 0.911$
Origin × Drought × Herbivory	***Mt***	$F_{1,762.1} = 0.9$ $p = 0.367$	$F_{1,656.1} = 1.0$ $p = 0.367$	$F_{1,657.5} = 0.8$ $p = 0.367$	**$Chi^2_1 = 9.1$ $p = 0.022$**	$F_{1,741.7} = 1.5$ $p = 0.367$	$F_{1,662.3} = 1.3$ $p = 0.367$	$F_{1,662.5} = 1.0$ $p = 0.367$	$F_{1733.5} = 0.9$ $p = 0.367$	$F_{1,366.0} = 1.2$ $p = 0.367$
	Pc	$F_{1,763.0} = 1.8$ $p = 0.912$	$F_{1,662.7} = 0.0$ $p = 0.912$	$F_{1,661.8} = 0.5$ $p = 0.912$	$Chi^2_1 = 0.0$ $p = 0.912$	$F_{1,757.9} = 1.2$ $p = 0.912$	$F_{1,702.1} = 0.0$ $p = 0.912$	$F_{1,701.5} = 0.0$ $p = 0.912$	$F_{1,754.5} = 0.8$ $p = 0.912$	$F_{1,369.7} = 0.0$ $p = 0.912$
	Cv	$F_{1,519.0} = 0.1$ $p = 0.947$	**$F_{1,450.8} = 6.4$ $p = 0.050$**	$F_{1,451.3} = 0.0$ $p = 0.947$	$Chi^2_1 = 1.0$ $p = 0.947$	$F_{1,508.9} = 0.0$ $p = 0.956$	$F_{1,487.3} = 0.4$ $p = 0.853$	$F_{1,487.2} = 1.9$ $p = 0.446$	$F_{1,507.0} = 0.3$ $p = 0.853$	$F_{1,297.8} = 1.7$ $p = 0.446$
	Lh	$F_{1,386.0} = 0.2$ $p = 0.911$	$F_{1,266.5} = 0.2$ $p = 0.911$	$F_{1,263.0} = 0.1$ $p = 0.911$	$Chi^2_1 = 3.6$ $p = 0.911$	$F_{1,347.4} = 0.3$ $p = 0.911$	$F_{1,278.0} = 0.1$ $p = 0.911$	$F_{1,256.0} = 0.0$ $p = 0.911$	$F_{1,347.6} = 0.1$ $p = 0.911$	$F_{1,256.4} = 7.5$ $p = 0.062$

Table S4 Number of used replicates for the analyses separated by the temporal origins (ancestors and descendants) and the four different treatments

Treatment	Temporal origin	Early size [kpixel]	Flowering onset	Size at flowering [cm]	Number of inflorescences	Vegetative biomass [mg]	Reproductive biomass [mg]	Reproductive investment	LDMC	Osmotic potential
Matthiola tricuspidata										
Control	Anc.	100	82	82	82	100	82	82	99	49
	Des.	100	99	99	99	100	99	99	100	50
Drought	Anc.	100	69	69	69	93	69	69	91	50
	Des.	100	95	95	95	95	95	95	92	49
Herbivory	Anc.	100	73	73	73	94	73	73	92	50
	Des.	100	97	97	97	98	97	97	97	51
Drought + Herbivory	Anc.	100	79	79	79	99	79	79	99	50
	Des.	100	99	99	99	100	99	99	100	50
Plantago crassifolia										
Control	Anc.	100	96	96	96	100	96	96	100	50
	Des.	100	91	91	91	100	91	91	99	50
Drought	Anc.	100	80	80	80	97	80	80	95	50
	Des.	100	82	82	82	99	82	82	99	50
Herbivory	Anc.	100	81	81	81	99	81	81	99	50
	Des.	100	78	78	78	99	78	78	98	50
Drought + Herbivory	Anc.	100	92	92	92	100	92	92	99	50
	Des.	100	97	97	97	100	97	97	100	50
Clinopodium vulgare										
Control	Anc.	72	60	60	60	71	60	60	71	45
	Des.	63	63	63	63	63	63	63	63	35
Drought	Anc.	72	59	59	59	67	59	59	67	45
	Des.	63	61	61	61	63	61	61	63	35
Herbivory	Anc.	73	54	54	54	72	54	54	72	45
	Des.	63	57	57	57	62	57	57	62	35
Drought + Herbivory	Anc.	72	57	57	57	71	57	57	71	45
	Des.	63	62	62	62	63	62	62	63	35
Leontodon hispidus										
Control	Anc.	49	47	47	47	49	47	47	49	35
	Des.	50	44	44	44	49	44	44	49	35
Drought	Anc.	49	24	24	24	36	24	24	36	32
	Des.	50	22	22	22	42	22	22	42	33
Herbivory	Anc.	49	28	28	28	45	28	28	44	36
	Des.	50	28	28	28	48	28	28	48	35
Drought + Herbivory	Anc.	49	45	45	45	49	45	45	48	34
	Des.	50	45	45	45	50	45	45	50	33

Synthesis

Human-induced climate change influences and challenges ecosystems worldwide. Its impacts started with the industrial revolution and accelerated dramatically during the last decades. Novel and more stressful environmental conditions threaten in particular plant species, which are sessile organisms, and individuals therefore have to cope with strong environmental fluctuations during their lifetime. Although plant populations have limited potential for migration, they can respond to changed environmental conditions through evolution of mean traits or of plasticity, which may only require a few generations (Franks *et al.* 2007; Carrol *et al.* 2007; Metz *et al.* 2020). **"Forward-in-time"** approaches, like experimental evolution or classic resurrection studies, are reliable methods to disentangle responses of plant populations to novel environmental conditions which are the result of adaptive evolution from plastic responses. This kind of research is crucial to detect the rate of phenotypic evolution and potential eco-evolutionary costs, to monitor responses to climate change and to improve knowledge to successfully apply evolutionary rescue, and restoration and conservation actions. However, such studies are either limited in time and/or space, lack the complexity of natural systems (Leuzinger *et al.* 2011; Kawecki *et al.* 2012), are resource intensive (Franks *et al.* 2018), and commonly consider only a single species and/or a few populations.

In this thesis, I aimed to overcome these drawbacks by using plants grown from ancestral seed bank material and comparing them to their contemporary populations. One goal of this thesis was to detect parallel patterns of recent evolution in European plant species in response to climate change. Another goal of this thesis, after developing and testing this novel approach, was to discuss its potential for future climate change research in plants and offer guidelines for following research projects. In **Chapter I**, I studied 13 species and investigated differences between ancestors and descendants with regard to phenology (flowering onset) and early growth to detect potential escape strategies to avoid summer droughts. I included the 6-year-IDM in the analyses to get a deeper insight into the impact of climate change on trait differentiation between the temporal origins. In **Chapters II** and **III,** I used watering treatments to mimic climate change and provoke potential differences between ancestors and their descendants. The treatments also served as a test of local adaptation, albeit under controlled greenhouse conditions (Kawecki and Ebert 2004). In **Chapter II**, using multiple species, I focused on trait differentiation in early life stages in response to drought, including shifts in both mean traits and plasticity. In contrast to the first two chapters, I used only four species in **Chapter III** which allowed me to increase precision by including more phenotypic and physiological measurements and to investigate interactions of

responses to drought with co-occurring herbivory. Furthermore, I disentangled selective from random processes driving recent trait changes by performing Q_{ST}-F_{ST} comparisons. Below, I summarize the results from my studies under the aspects of 1) advanced phenology of contemporary populations and 2) how these phenological shifts might influence the persistence of populations in natural environments. I further discuss 3) the potential of seed banks to adapt the resurrection approach, including some guidelines for future studies, and 4) suggest potential follow-up projects within this field of work.

Advanced phenology of contemporary populations

My studied populations originated from regions where climate change within the last decades led to more frequent and severe droughts during the growing season in spring and summer (see General introduction). There is mounting evidence that a shortening of plants‘ life cycles through rapid growth and early flowering is an appropriate strategy to avoid drought stress and to ensure population survival (Franks *et al*. 2007; Kigel *et al.* 2011; Metz *et al*. 2020). I found strong evidence that the descendants grew faster within the first three weeks and flowered earlier (**Chapter I**). These results were confirmed by and complemented with evidence on shifts in resource allocation in **Chapter III**. In addition to this general difference between ancestors and their descendants, I demonstrated that precipitation regimes and drought risk promote advanced flowering (see **Chapter I**). Besides this relationship between climatic conditions and flowering, the Q_{ST}-F_{ST} comparisons in **Chapter III** showed for *Matthiola tricuspidata*, *Clinopodium vulgare* and *Leontodon hispidus* that flowering onset was under directional selection instead of random processes such as drift. These findings are in line with other studies showing highest Q_{ST} values, indicating strong selection, for flowering-related traits (Chun *et al.* 2011; Kesselring *et al.* 2015). In order to strengthen these analyses, future experiments investigating evolution in plant populations over time should incorporate more populations per species to increase the reliability of the Q_{ST} calculations (Leinonen *et al.* 2013) and to assess natural genetic variation in space and time. Furthermore, Q_{ST}-F_{ST} comparisons could be complemented with calculations of selection differentials (Parachnowitsch and Kessler 2010) or genome-wide scans to detect genes that were under selection (Rhoné *et al.* 2010; Frachon *et al.* 2017). In contrast to some species in **Chapter I** and **III,** the seedling survival experiment in **Chapter II** showed that the descendants grew slower than their ancestors, which would indicate a delay in the plants‘ development. It is known that the timing of water deficits during a plant’s life cycle may influence growth and stress reactions (Rozijn and Van der Werf 1986; Kron *et al.* 2008), which could lead to the different responses observed in **Chapter I** and **III** in comparison to **Chapter II**. Furthermore, this result in **Chapter II** was strongly

influenced by *Anthemis maritima*, which was the only species in **Chapter I** for which ancestors flowered earlier and grew faster within the first three weeks.

Although the cross-species analyses (**Chapter I**) and three out four species (**Chapter III**) indicated the above-mentioned differentiations between the temporal origins, some species showed opposite results. For example, for *A. maritima* (**Chapter I**) or *L. hispidus* (**Chapter III**) descendants showed delayed flowering in comparison to their ancestors. I assert that all sites of origin vary in their edaphic, microclimatic and biotic environments, all of which might have experienced changes during the last decades. For *L. hispidus* – growing in grasslands – alterations in the management strategies within the last 20 years, which may select more strongly for flowering onset than climatic changes (Völler *et al.* 2012), could explain why the descendants flowered later than their ancestors. For the beach species of *A. maritima* it is more difficult to find a good explanation for its delayed flowering.

Consequences of phenology shifts in natural environments

I performed all my experiments under controlled common garden conditions in the greenhouse and only manipulated at most two environmental factors simultaneously. This method was useful to detect parallel patterns of differentiations between contemporary and ancestral populations among species but did not come near the complex environments that plants face under natural conditions. Advanced flowering of contemporary populations in comparison to their ancestors could safeguard their survival under future climate change with an increased risk of droughts. In addition, shortening of life cycles can also be advantageous to escape from other environmental stressors like insect herbivore outbreaks (see **Chapter III**; Pilson 2000; Kawagoe and Kudoh 2010). However, populations with accelerated life cycles are at higher risk of experiencing late-frost damage (Zohner *et al.* 2020), mismatches with pollinators during flowering (Hegland *et al.* 2009; Thackeray *et al.* 2016) or potential trade-offs with trait responses to co-occurring environmental stressors (Pilson 2000). Furthermore, especially in regions where climate change leads to more unpredictable environmental conditions (Altvater *et al.* 2011), plant populations should also show stronger plasticity (Ackerly *et al.* 2000; Richards *et al.* 2006) or higher genetically based phenotypic variability (Karbstein *et al.* 2019, 2020) to lower the risk of local extinction. In my experiments, I found evidence for higher plasticity in the descendants indicated by stronger responses in plant size to the watering treatment in **Chapter II** and for higher phenotypic variability in **Chapter I** by the trend that contemporary and drier populations had higher CVs for flowering onset. To test whether the observed shifts in phenology, either in the mean or in the strength of

plasticity, were adaptive requires transplant experiments at the species' sites of origin to include the environmental complexity and to test for local adaptation (Kawecki and Ebert 2004). In addition, these experiments should ideally incorporate long-term measurements of plant fitness (Richards *et al.* 2006).

Seed banks – A untapped resource for climate change research?

I adapted the **"forward-in-time"** resurrection approach by comparing ancestral seed bank material with freshly sampled seeds from the same populations, thereby turning it into a **"back-in-time"** approach. In contrast to the guidelines for resurrection studies recommended by Franks and colleagues (2018), seed bank material has not been collected on purpose to conduct evolutionary research and thereby potentially comes along with some methodological difficulties and uncertainties that I addressed (see General Introduction p. 13–15).

First, the collection procedures of the ancestral lines are often unknown and could therefore represent an unrepresentative sub-sample of the populations' genetic and phenotypic diversity. Concerning this I mentioned in the general introduction, that the number of seeds per accession in the seed banks was high, which is a reasonable proxy for a sufficient amount of collected individuals. Furthermore, collectors were already aware in the past that ex-situ collections have to represent the genetic diversity of the populations (Brown 1989; Falk and Holsinger 1991; Guarino 1995; Way 2003). In addition, unintentional selection during sampling and/or invisible fractions through different or very low germination rates (Weis 2018) might cause observable differentiations between the two temporal origins. In theory, orthodox seeds can survive dried and frozen in seed banks for tens to hundreds of years (Walters *et al.* 2005; Liu *et al.* 2018; Solberg *et al.* 2020). I found that the ancestral collections generally showed lower germination rates (**Chapter I**) but large differences with germination of descendants were only present in three species (*Medicago marina*, *Plantago subulata* and *L. hispidus*).

To assess the impact of unintentional selection during sampling and invisible fraction after germination, I used molecular data ddRAD-SNP marker (see Box 2, p. 46–48) comparing the relative genomic relatedness of ancestors to that of descendants. I showed that the relatedness of plants is similar within ancestors and descendants for 12 out of the 18 studied species providing further support for sampling of roughly similar (or alternatively: high) numbers of maternal plants and similar genetic variability. Thus, indicating similar sampling methods and no or negligible selective processes during storage and germination. Furthermore, the results of my multi-species experiments (**Chapter I** and **Chapter II**, "watering response experiment") were consistent across many species, which is very unlikely if part or all of these patterns were due to random processes

like drift or gene flow.

Besides the above discussed uncertainties, inherent in my work, two of my experiments (**Chapter I** and **Chapter II**) are lacking refresher generations, which is often discussed as mandatory to standardise material from different spatial or temporal origins by reducing maternal and storage effects (Franks *et al.* 2018, discussed in **Chapter I**). Concerning this, I found consistency in flowering time within species when I compared the results from **Chapter I** (F0 generation) and **Chapter III** (F1 generation, after refresher generation). Descendants of *C. vulgare* and *M. tricuspidata* showed advanced flowering, whereas descendants of *L. hispidus* showed delayed flowering in both experiments. This consistency may indicate that at least for these three species the potential maternal and storage effects have weak effects on later life stages.

However, with regard to germination rates I also detected differences between F0 (**Chapter I**) and F1 (**Chapter III**). In *C. vulgare* and *M. tricuspidata* germination rates in both temporal origins were lower and for *Plantago crassifolia* higher in F1 in comparison to F0. For *L. hispidus* we found a large difference in germination between the temporal origins (higher rates in the descendants) in the F0 generation, which decreased in F1. I explain these inconsistencies between the different generations with the collection of immature seeds (all species), seed mortality during storage in the seed bank (*L. hispidus*) or with inefficient hand pollination in the greenhouse (*C. vulgare* and *M. tricuspidata*). In general, my findings are in line with other studies showing stronger impact of maternal effects on germination and young seedlings and weaker influence on later life stages (Baskin and Baskin 2004; Hereford and Moriuchi 2005; Bischoff and Schärer 2010).

Overall, I am confident that seed bank collections are an untapped resource for climate change research and can be used to turn the **“forward-in-time”** resurrection approach into a **“back-in-time”** approach. Apparent uncertainties, like a potential unequal representation of the temporal origins‘ genetic and phenotypic diversity, can be minimised by a well considered selection of target species and populations, as well as by properly conducted experiments. Refresher generations, if unfeasible, can be omitted, which enables the inclusion of a large amount of species from different taxa in multi-species experiments to detect general patterns (van Kleunen *et al.* 2014). In all cases, experiments demand a high degree of preparation. First, researchers should select species with known ecology. The required conditions for germination should be achievable under greenhouse conditions, with high germination rates. Furthermore, precise information about the populations’ origin has to be provided by the seed banks and collaborations with local experts are important to reduce effort during relocation and sampling. In

Figure 1, I illustrated potential working steps for future studies using seed bank collections adapting the resurrection approach. This suggested procedure is the result of my experiences during this project and the findings I discussed above.

After scanning the catalogues of five different European seed banks, I had chosen 95 populations but ended up with 18 populations that I investigated in this thesis. Reasons for this low success rate (19%) include difficulties in relocating populations and in collecting seeds, partial low germination rates within at least one of the temporal origins, and finally challenges during cultivation in the greenhouse (no flowering over two years). This is why I recommend including as many species and populations as possible at the beginning of a multi-species research project. Ideal species for such studies should be easy to cultivate, should flower after one generation and should have a high seed set to enable broad experimental possibilities. Good examples of study species from my initial selection turned out to be *C. vulgare* and *M. tricuspidata,* which I ended up using in all three chapters.

Summary and Outlook

In this thesis, I developed and tested a new method for investigating evolutionary shifts in plant populations over time. On the one hand, in contrast to regular **"forward-in-time"** resurrection studies (Franks *et al.* 2018), my approach using seed bank collections has several drawbacks such as uncertain sampling procedures of the ancestors or potential selection during storage. However, I showed that these apparent uncertainties can be minimised by accurate planning and execution of the experiments. On the other hand, using seed bank resources enables us to perform powerful multi-species experiments aimed at uncovering parallel patterns of evolution. I investigated European plant species which experienced more frequent and severe droughts during the growing season within the last two to three decades. I found evidence for "escape strategies" indicated by advanced life cycles of the contemporary populations in comparison to their ancestors (**Chapter I** and **Chapter III**), which is likely the result of selection rather than random evolutionary processes (**Chapter III**). I further showed evidence for increased plasticity of the descendants in response to drought (**Chapter II**, "watering response experiment") and evidence for higher drought resistance (**Chapter II**, "seedling survival experiment" and **Chapter III**).

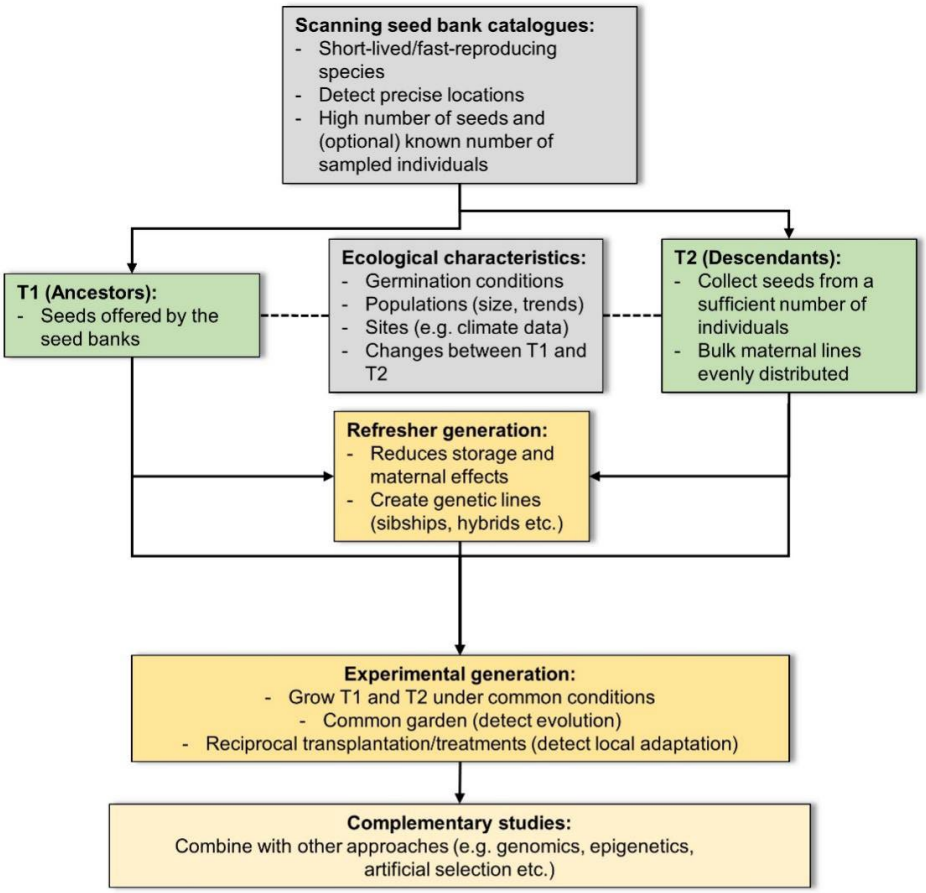

Figure 1 A flowchart of example procedures for using seed banks collections adapting resurrection studies comparing ancestors and descendants to study evolution, including recommendations for best practices. See text for further details.

Given that every ecological study is resource-limited, researchers have to consider trade-offs between generality, precision and realism (Fig. 2). Whereas regular resurrection studies are very precise in elucidating evolutionary processes in a single or few populations of a single species, my experiments aimed to find general patterns across species with – relatively speaking – one study focusing on generality (**Chapter I**, multi-species without treatments), another on realism (**Chapter II**, watering treatment), and a third on precision as well as realism (**Chapter III**, using F1 and treatment combinations). Understandably, this **"back-in-time"** method cannot replace classical resurrection approaches or well-designed, long-term collections of many species and populations for future evolutionary research like the "Project Baseline" (Etterson *et al.* 2016, Fig. 2 "A"). However, the use of seed bank collections broadens up the possibilities for research

on global environmental changes and their impacts on plant populations. There are thousands of properly sampled, frozen ancestral accessions with known locations stored in seed banks; researchers could use these resources in combination with molecular methods to investigate phenotypic and genetic shifts in populations across spatial and, if accessions from different time points from the same location are available, temporal gradients (Fig. 2 “B“). Future studies should also include transplanting experiments of ancestors and descendants to the original habitats to incorporate the entire and natural environmental complexity and to test for local adaptation of the descendant compared to the ancestor population (Fig. 2 “C“). Future studies should also include long-term fitness measurements in perennial plants (Fig. 2 “C“). Additionally, if data suggest or confirm that seed bank collections and freshly collected seeds represent similar genetic variability, population-specific experiments can be conducted with locality-specific treatments or hybridisations between ancestors and descendants to investigate the genetic basis and architecture of trait changes (Fig. 2 “D“). Environmental conditions changed rapidly across the globe over the last few decades and will increasingly change in the future. Experiments studying plants‘ evolutionary responses will help to detect potential trade-offs of adaptation, to monitor responses to global change and to gain knowledge of evolutionary rescue, restoration and conservation, which is crucial to preserve biodiversity.

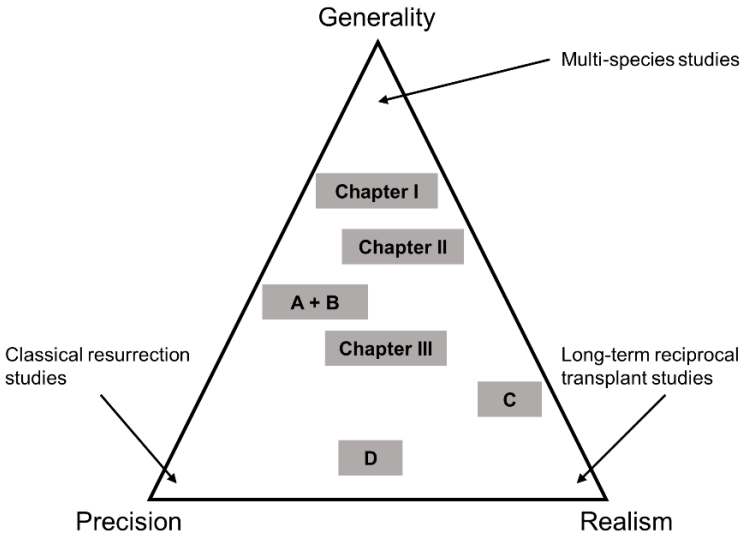

Figure 2 Trade-offs between “Precision”, “Generality” and “Realism” in ecological experiments due to limited resources including the positions of the chapters from this thesis and suggested future experiments (A – “Project Baseline”, B – multi-populations, C – transplant experiments using ancestors and descendants, D – single population experiments)

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Appendix

Methods: ddRAD library preparation, SNP genotyping and population genomic analyses

We collected leaf samples from plants grown in a common garden and freeze-dried them. After DNA extraction using the 'peqGOLD Plant DNA Mini Kit' (VWR peqlab, Darmstadt, Germany) and DNA quantitation with Qubit (Thermo Fisher Scientific) we followed the ddRAD protocol by (Peterson *et al.* 2012) with minor modifications, using 100 ng DNA per sample and *EcoRI* and *MspI* as restriction enzymes to generate 12 ddRAD libraries comprising 516 samples. Libraries were pooled equimolarly and sequenced (PE, 150bp) on four lanes of an Illumina HiSeq2000, resulting in a total of $7.61*10^8$ sequences.

We used process_radtags from the Stacks 2.0 pipeline (Rochette *et al.* 2019) to demultiplex reads. Sequence data have been deposited in the European Nucleotide Archive (ENA) at EMBL under accession number PRJEB47887 with individual accession numbers ERS7667629 to ERS7668109. Subsequently, we used *dDocent* 2.6.0 (Puritz *et al.* 2014) to assemble reads and call SNPs. We set *Clustering_Similarity%* to 0.88, minimum within individual coverage level to include a read for assembly (K1) to 5, minimum number of individuals a read must be present in to include for assembly (K2) to 6 and default values for other parameters. Although the species have different ploidy levels (7 and 6 species are di- and tetra-ploid, respectively) we assumed diploidy for all species because this allowed an identical data analysis across species. Thus, we identified between 1,163,740 and 1,290,150 raw SNPs across species and filtered these raw SNPs following (O'Leary *et al.* 2018). First, we used the functions vcfallelicprimitives and vcftools to remove indels, keeping only biallelic SNPs with minimum allele count of 3 (*mac* 3), minimum genotype read depth of 3 (*minDP* 3), minimum mean sequence quality of 30 (*minQ* 30), maximum missingness across individuals of 50% (*max_missing* 0.5) and skipping individuals with >75% missing values (*imiss* > 0.75). Subsequently, using vcffilter, we filtered SNPs according to allele balance, strandedness, mapping quality ratio of the two alleles, and status of properly pairing of alleles using parameter values. Using vcftools, we then filtered SNPs to maximum missingness of 33% (*max_missing* 0.66), minimum minor allele frequency of 0.05, minimum mean read depth of 20 and maximum mean depth of 1000. In the end we retained only a single SNP per contig. After import into R, we further filtered SNPs to maximum missingness of 30% using gl.filter.callrate (threshold = 0.70) from the dartR package (Gruber *et al. 2017)*. The final data sets consisted of between 11 and 20 per species, genotyped at between 204 and 6028 (average 3255) biallelic SNP loci.

We assessed pairwise genomic relatedness among samples within ancestral and descendant populations using two estimators, genomic relatedness *G* (Yang *et al.* 2010) through function dartR::gl.grm and the kinship estimator r^{β} (Goudet *et al.* 2018), through function beta.coan.SNPs available at https://datadryad.org/stash/dataset/doi:10.5061/dryad.ds8fk04,
which were applied to ancestral and descendant individuals in one population. Both estimators are relative measures of relatedness based on genomic marker data which take a value of zero for pairs of randomly related individuals, positive values for more closely and negative values for less closely related than expected at random given allele frequencies of the population. For each species, we tested for significant differences of pairwise relatedness between ancestral and descendant populations using analysis of variance (aov).
In addition we assessed genomic diversity within ancestral and descendant populations as allelic richness, thus correcting for differences in sample size by rarefaction (Ar, El Mousadik and Petit 1996), with the function allel.rich, and the number of private alleles with the function gl.report.pa, both from the R-package PopGenReport (Adamack and Gruber 2014). We quantified neutral genetic differentiation between ancestral and descendant populations as pairwise F_{ST} using the function stamppFst from the R-package StAMPP (Pembleton *et al.* 2013).

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Table A1 Overview of all planned species and populations of the five seed banks fulfilling the criteria for an usage in resurrection approach experiments using the “back-in-time“ mode. CBNMed – Conservatoire Botanique National Méditerranéen de Porquerolles, CBN-Alpin – Conservatoire Botanique National Alpin de Gap-Charance, MBG – Meise Botanic Garden, BGO – Botanical Garden of the University of Osnabrück, BBG – Berlin Botanic Garden and Botanical Museum; Status: not found – I could no relocate the population, less – either to less seeds or to low germination rates, not used – no usage in this work, used – populations which are included in the experiments of this work

Species	Family	Year of collection	Status
CBNMed, France			
Allium chamaemoly	Amaryllidaceae	1991	not found
Ammophila arenaria	Poaceae	1994	not used
Anthemis maritima	Asteraceae	1992	used
Anthyllis barba-jovis	Fabaceae	1992	not used
Artemisia arborescens	Asteraceae	1982	not found
Aster tripolium	Asteraceae	1991	not found
Brassica montana	Brassicaceae	1992	not found
Convolvulus soldanella	Convolvulaceae	1995	less
Crucianella maritima	Rubiaceae	1994	not found
Echinophora spinosa	Apiaceae	1995	not found
Elytrigia juncea	Poaceae	1994	used
Euphorbia peplis	Euphorbiaceae	1998	less
Hyoscyamus albus	Solanaceae	1983	not found
Matthiola tricuspidata	Brassicaceae	1994	used
Medicago marina	Fabaceae	1992	used
Pallenis maritima	Plantaginaceae	1994	less
Plantago crassifolia	Plantaginaceae	1994	used
Plantago subulata	Plantaginaceae	1997	used
Pseudorlaya pumila	Apiaceae	1992	less
Silene nicacensis	Caryophyllaceae	1980	less
CBN-Alpin, France			
Aconitum anthora	Ranunculaceae	1997	not found
Allium narcissiflorum	Amaryllidaceae	1997	less
Aquilegia alpina	Ranunculaceae	1997	less
Berardia subacaulis	Asteraceae	1997	less
Carduus aurosicus	Asteraceae	1997	less
Cicerbita alpine	Asteraceae	1997	less
Eryngium spinalba	Apiaceae	1997	less
Iberis aurosica	Brassicaceae	1997	less
Lotus alpinus	Fabaceae	1991	less
Papaver alpinum	Papaveraceae	1997	less

Table A1 continued

Species	Family	Year of collection	Status
Potentilla delphinensis	Rosaceae	1997	less
Primula halleri	Primulaceae	1991	not found
Primula hirsuta	Primulaceae	1997	less
Pulsatilla halleri	Ranunculaceae	1998	not found
Rhaponticum heleniifolia	Asteraceae	1997	less
Thalictrum aquilegiifolium	Ranunculaceae	1997	less
Trifolium alpinum	Fabaceae	1991	less
Trifolium aureum	Fabaceae	1998	less
MBG, Belgium			
Angelica sylvestris	Apiaceae	1991	not found
Anthyllis vulneraria	Fabaceae	1992	less
Bistorta officinalis	Polygonaceae	1991	less
Bupleurum falcatum	Apiaceae	1992	not found
Carex flacca	Cyperaceae	1992	less
Centaurium erythraea	Gentianaceae	1992	used
Clinopodium vulgare	Lamiaceae	1992	used
Digitalis lutea	Scrophulariaceae	1993	less
Digitalis lutea	Scrophulariaceae	1993	less
Digitalis lutea	Scrophulariaceae	1992	used
Digitalis purpurea	Scrophulariaceae	1991	less
Globularia bisnagarica	Globulariaceae	1992	used
Leontodon hispidus	Asteraceae	1995	used
Melica ciliata	Poaceae	1992	used
Pimpinella saxifraga	Apiaceae	1992	used
Potentilla neumanniana	Rosaceae	1992	less
Rhinanthus minor	Scrophulariaceae	1995	less
Rhinanthus minor	Scrophulariaceae	1991	less
Rhinanthus minor	Scrophulariaceae	1991	less
Sanguisorba minor	Rosaceae	1992	used
Scabiosa columbaria subsp. columbaria	Dipsacaceae	1992	less
Sedum album	Crassulaceae	1992	used
Silene nutans	Caryophyllaceae	1992	not found
Silene nutans	Caryophyllaceae	1992	not found
Teucrium chamaedrys	Lamiaceae	1992	used
BGO, Germany			
Aethusa cynapium	Apiaceae	1994	not found
Agrimonia eupatoria	Rosaceae	1994	not found
Amaranthus retroflexus	Amaranthaceae	1994	not found
Anthyllis vulneraria	Fabaceae	1995	not found
Caltha palustris	Ranunculaceae	1994	less

Table A1 continued

Species	Family	Year of collection	Status
Cirsium acaule	Asteraceae	1994	not found
Dianthus carthusianorum	Caryophyllaceae	1993	less
Echium vulgare	Boraginaceae	1996	not found
Echium vulgare	Boraginaceae	1996	not found
Gentianella germanica	Gentianaceae	1993	not found
Hypericum pulchrum	Hypericaceae	1995	not found
Hypericum montanum	Hypericaceae	1997	less
Jasione montana	Campanulaceae	1993	not found
Juncus conglomeratus	Juncaceae	1993	not found
Lithospermum officinale	Boraginaceae	1996	less
Peucedanum palustre	Apiaceae	1996	not found
Rorippa palustris	Brassicaceae	1993	not found
Sanguisorba minor	Rosaceae	1995	less
Sanicula europaea	Apiaceae	1995	not found
Saxifraga tridactylites	Saxifragaceae	1997	not found
Sisymbrium altissimum	Brassicaceae	1988	not found
Solanum dulcamara	Solanaceae	1995	not found
Verbascum nigrum	Scrophulariaceae	1995	not found
Veronica arvensis	Scrophulariaceae	1997	not found
BBG, Germany			
Angelica archangelica	Apiaceae	1994	not found
Dianthus superbus	Caryophyllaceae	1987	not found
Helianthemum nummularium	Cistaceae	1987	not found
Silene chlorantha	Caryophyllaceae	1980	used
Stipa capillata	Poaceae	1995	not found
Swertia perennis	Gentianaceae	1991	not found
Trollius europaeus	Ranunculaceae	1991	not found
Verbascum densiflorum	Scrophulariaceae	1985	not used