Ecological and Evolutionary Relevance of Plant Responses to Environmental Variability

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Contents of this thesis

Declaration of author contributions

The thesis entitled ”Ecological and evolutionary relevance of plant responses to environmental variability” is based on the work I did during my PhD at University of Tübingen, supervised by Prof. Dr. Oliver Bossdorf, and under the collaboration with Dr. J.F. (Niek) Scheepens and Dr. Madalin Parepa. In this thesis, chapters II - V include four 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 II

YD, JFS, MP and OB designed the experiment. YD conducted the experiment, collected data and performed data analysis. JFS, MP and OB supervised data analysis and interpretation of the results. YD wrote the manuscript with input from MP and OB.

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Chapter III

YD, JFS, OB and MP designed the experiment. YD performed the experiment, collected data and conducted data analysis. JFS, MP and OB supervised the analysis and interpretation of the results. YD wrote the manuscript with input from JFS, OB and MP.

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Chapter IV

JFS and OB designed the experiment. JFS and YD performed the experiment. JFS analysed the data and drafted the manuscript. YD and OB contributed to the final version of the manuscript.

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Chapter V

YD, JFS and OB designed the experiment. YD performed the experiment and analysed the data. YD wrote the manuscript with input from OB and JFS.

Status in publication process: in preparation

"Glaciers are melting, sea levels are rising, cloud forests are dying, and wildlife is scrambling to keep pace… All of those changes are emerging as humans continue to add heat-trapping greenhouse gases to the atmosphere, changing the rhythms of climate that all living things have come to rely on."

Christina Nunez, 2019, National Geographic

Abstract

We are the witnesses of a drastic planetary environmental change. Recent climate change caused by anthropogenic activities has exceeded the boundary of natural variation and influences life on earth, and organisms respond to this global change through range shifts, phenological responses and evolutionary changes. Besides the well-known changes in environmental means, we also see trends of increasing temporal environmental variability, for instance through increased frequencies and intensities of extreme weathers and resulting changes in other environmental variables such as soil resource fluctuations.

Terrestrial plants can respond to environmental change in different ways, from short-term phenotypic plasticity, to cross-generational responses and long-term population changes and community turnover. The latter two are driven by genetic and species differences in plant environmental responses, respectively. Thus, to understand and predict the effects of environmental change, we not only need to study the plastic responses of plants, but also their intra- and interspecific variation in this respect. Some previous studies on the ecological effects of increased climatic and environmental variability have shown that changes in environmental variability can have substantial effects on plant communities and ecosystems, but there are still many open questions. This thesis addresses several gaps in our understanding of plant responses to increased environmental variability: (i) What are the effects of increased environmental variability *per se* on plants, and how do these effects compare to those of changes in environmental means? (ii) What is the relative importance of different components of environmental variability, in particular the timing versus frequency of environmental fluctuations? (iii) How do plants plastically respond to increased environmental variability across generations? (iv) How much variation is there among different plant species and genotypes within species, and does this variation reflect their ecological origins and evolutionary history?

In my thesis, I present a set of ecological experiments in which I tested these questions in short-lived plants. These plants are particularly suitable for experiments because of their sensitivity to short-term changes in climate and environmental variability, and because their fitness can be easily assessed. Chapters II & III include two experiments with experimental nutrient fluctuations in which I compared the overall effects of changes in temporal nutrient variability to effects of changes in nutrient means, with chapter II investigating both among- and within-species variation in a set of common European annual plants, each from multiple geographic origins, and chapter III focusing on a much broader comparison of 37 annual species, with a test for a phylogenetic signal in their nutrient variability responses. Chapters IV & V present a second set of two related experiments in which I compared the effects of timing versus frequency of temperature fluctuations in different *Arabidopsis thaliana* genotypes across two generations, with chapter IV investigating the genotypic variation in responses to temperature stress in the maternal plants, and chapter V investigating the transgenerational effects on their offspring. In both generations, I also tested for relationships between genotypes' responses and their climate of origin, as potential indication for adaptive significance.

My results show that: (i) Changes in environmental variability affect plants, but the magnitude of these effects depends on the environmental mean. (ii) Different aspects of environmental variability have different effects on plants. In the case of the temperature treatments tested in my experiments, the timing of temperature stress had much stronger

impacts on plants than its frequency. (iii) The plastic responses of plants to environmental variability can be expressed in the following generation. Also here I found much stronger effects of timing of (parental) temperature stress than frequency of (parental) temperature stress on offspring performance. (iv) There is significant variation in plant responses to increased environmental variability both among plant species and among genotypes within the same species. The variation among species can be partly explained by their shared phylogeny, while variation within species is related to the climatic variability of their geographic origins, indicating a possible adaptive significance. Together, my findings suggest that there is both ecological and evolutionary relevance in plant responses to increased environmental variability, and that changes in environmental variability will result in plant population and community changes. Future studies of global change effects on plant species should attempt to separate effects of environmental variability from those of environmental means. Moreover, long-term experiments and field studies should test the predictions from short-term and simplified lab experiments. This will help us to better understand global change effects on natural ecosystems.

Zusammenfassung

Wir sind Zeugen der voranschreitenden globalen Umweltveränderungen. Der jüngste Klimawandel infolge anthropogener Aktivitäten hat die Grenzen natürlicher Variation überschritten und beeinflusst Lebewesen aller Art. Auf Grund dieses globalen Wandels kommt es zu Verbreitungsverschiebungen, phänologische Reaktionen und evolutionären Veränderungen bei vielen wilden Arten. Neben den allgemeinen bekannten Veränderungen klimatischer Durchschnittswerte, zeichnen sich Trends hin zu einer höheren zeitlichen Variabilität der Umwelt, beispielsweise via steigender die Häufigkeit und Intensität extremer Wetterereignisse zu, die wiederum anderer Umweltvariablen – beispielsweise Nährstoffverfügbarkeit in Böden – beeinflussen.

Terrestrische Pflanzen können auf Umweltveränderungen auf verschiedene Weise reagieren, mit kurzfristiger phänotypischer Plastizität, generationenübergreifenden Reaktionen, oder langfristigen Veränderungen von Populationen und Pflanzengemeinschaften. Die Ursache für die letzten beiden Optionen liegen in genetischen bzw. artspezifischen Unterschieden in Bezug auf die Reaktion auf Umweltvariabilität. Um die Folgen des Globalen Wandels vorherzusagen und zu verstehen, ist es daher nicht nur nötig die plastischen Reaktionen von Pflanzen zu studieren, sondern auch ihre intra- und interspezifischen Unterschiede in dieser Hinsicht. Vergangene Studien über die ökologischen Effekte steigender Klima- und Umweltvariabilität haben gezeigt, dass Veränderungen der Umweltvariabilität substanzielle Folgen für Pflanzengesellschaften und Ökosysteme haben können. Dennoch gibt es noch viele offene Fragen. Diese Studie thematisiert einige der Wissenslücken in unserem Verständnis der Reaktion von Pflanzen auf steigende Umweltvariabilität: (i) Wie wirkt sich eine erhöhte Umweltvariabilität *per se* auf Pflanzen aus und in welchem Verhältnis steht sie zu veränderten Umweltmittelwerten? (ii) Welche relative Bedeutung haben die verschiedenen Komponenten der Umweltvariabilität, im Besonderen das Timing versus die Frequenz von Umweltfluktuationen? (iii) Wie reagieren Pflanzen plastisch auf steigende Umweltvariabilität, über mehrere Generationen? (iv) Wie viel Variation gibt es zwischen verschiedenen Pflanzenarten und Genotypen innerhalb einer Art, und spiegelt diese Variation ihre evolutionäre und ökologische Geschichte wider?

In meiner Arbeit präsentiere ich eine Reihe von ökologischen Experimenten, mit denen ich diese Fragen in kurzlebigen Pflanzen getestet habe. Diese Pflanzen sind hierfür gut geeignet, da sie sensitiv auch auf kurzzeitige Klima- und Umweltschwankungen reagieren und sich ihre Fitness leicht evaluieren lässt. Kapitel II & III beinhalten zwei Nährstofffluktuationsexperimente, in denen ich die Gesamtwirkung der Veränderung der zeitlichen Variabilität der Nährstoffvariabilität mit derjenigen der mittleren Nährstoffverfügbarkeit vergliche. Kapitel II thematisiert die Variabilität zwischen und innerhalb einer Reihe von häufigen, europäischen, einjährigen Pflanzenarten verschiedener Herkunft. Kapitel III konzentriert sich auf einen breiten Vergleich der zwischenartlichen Variationen von 37 einjährigen Arten und einer möglichen phylogenetischen Assoziation ihrer Reaktion auf Nährstoffvariabilität. Kapitel IV & V präsentieren ein zweites Set verwandter Experimente, in denen ich die genotypische Variation in Reaktion auf Temperaturstress in verschiedenen *Arabidopsis thaliana* Genotypen über zwei Generationen verglichen habe. Kapitel IV untersucht die genotypische Variation in den Reaktionen auf Temperaturstress bei maternalen Pflanzen, und Kapitel V behandelt die generationsübergreifenden Effekte auf ihre Nachkommen. In beiden Generationen habe ich

die Korrelation zwischen den Reaktionen der Genotypen und den klimatischen Bedingungen ihres geographischen Herkunftsgebietes als potenziellen Nachweis für eine Anpassung getestet.

Meine Experimente zeigen: (i) Veränderungen der Umweltvariabilität beeinflussen Pflanzen, aber das Ausmaß dieser Effekte ist abhängig vom Durchschnittswert der Umweltvariable. (ii) Die verschiedenen Komponenten der Umweltvariabilität können unterschiedliche Auswirkungen auf die Reaktionen der Pflanzen haben, im Falle von Temperaturveränderungen hat der Zeitpunkt und nicht die Häufigkeit von Temperaturstress einen deutlich stärkeren Einfluss auf die Pflanzen. (iii) Die plastischen Reaktionen von Pflanzen auf Umweltvariabilität können sich in der folgenden Generation manifestieren. Für die Fitness der Nachkommen hatte dabei der Einfluss des Timings des (elterlichen) Temperaturstress einen größeren Einfluss als dessen Frequenz. (iv) Es gibt eine signifikante Variation in der Reaktion von Pflanzen auf steigende Umweltvariabilität – sowohl zwischen Arten als auch zwischen der Genotypen innerhalb einer Art. Die Unterschiede zwischen den Arten lassen sich teilweise durch ihre gemeinsame Phylogenie erklären, während Variation innerhalb der Arten verbunden ist mit der klimatischen Variabilität ihrer geographischen Herkunft, was auf eine mögliche adaptive Bedeutsamkeit hindeutet. Zusammengenommen deuten meine Ergebnisse darauf hin, dass die Reaktionen von Pflanzen auf erhöhte Umweltvariabilität sowohl ökologische als auch evolutionäre Relevanz hat und dass Veränderungen der Umweltvariabilität dazu führen das sich Pflanzengesellschaften und -populationen verändern. Weitere Studien über die Auswirkungen von globalem Wandel auf Pflanzenarten sollten versuchen die Effekte von Umweltvariabilität und die der Durchschnittswerte der Umweltvariablen zu trennen. Weiterhin sollten Langzeit-Experimente und Feldstudien die Vorhersagen die in Kurzzeit- und vereinfachte Laborexperimente gemacht wurden überprüfen. Dies wird uns helfen den Einfluss des globalen Wandels auf natürliche Ökosysteme besser zu verstehen.

Chapter I

General introduction

General introduction

Our planet Earth is facing a great challenge, an ongoing process we call "global change". From historical records and modern-day observations we see a rapidly growing human influence on the global environment (Vitousek 1992). Through the release of greenhouse gases and land surface alterations, humans are causing global environmental changes that exceed the boundaries of natural variation (Meyer and Turner 1994; Karl and Trenberth 2003). To date, climate warming is on average approximately 1.0°C above pre-industrial levels and will reach a global average increase of 1.5°C by the mid-21st Century. Global land precipitation has increased by about 2% since the beginning of the 20th century (Houghton et al. 1996; Hulme et al. 1998; IPCC 2018).

Such global environmental changes have a direct and permanent influence on the life on Earth. Across the globe, biotic responses to global warming are well recorded, from species range shifts and phenological changes to evolutionary responses (Walther et al. 2002; Parmesan and Yohe 2003; Root et al. 2003; Parmesan 2006). For instance, species are moving both towards higher elevations and higher latitudes at an average rate of 11.0 meters and 16.9 kilometers per decade, respectively, in order to track climate warming (Chen et al. 2011). Within their ranges, species are responding to regional climate change both phenotypically and genetically. In terrestrial plants, early onset of spring events is reported across different regions and continents (Fitter and Fitter 2002; Menzel et al. 2006). There is also evidence of evolutionary responses through altered genetic composition (Bradshaw and Holzapfel 2006; Parmesan 2006; Merilä 2012), for instance the increased frequency of heat tolerant genotypes as adaptation to climate warming (Parmesan 2006). When species fail to move, shift their phenotypes or adapt to rapid climate change, they have a high risk of population decline and to go extinct, as there is already a high record of biodiversity loss globally (Ceballos et al. 2017).

Most discussion and research on global change is currently centered around particular changes in the means of environmental factors, e.g. increased temperature or nitrogen deposition. However, this is not the whole story of global change. Recently, scientists have also recognized trends of increasing climatic variability accompanied by more frequent and intense weather extremes, such as heat waves and heavy rainfalls, and these trends are likely to continue in the future (Groisman et al. 1999; Folland et al. 2001; Meehl and Tebaldi 2004; Dore 2005; Min et al. 2011). In fact, recurrent extreme European hot summers (e.g. in 2003 & 2010) are expected in the coming decades, and temperature variability is predicted to continue to increase (Schär et al. 2004; Fischer and Schär 2009; Barriopedro et al. 2011).

Extreme events that follow from such increased climatic variability can have large repercussions for populations and ecosystems (Jentsch et al. 2007). Given that the impacts of short-term extreme events can be significant, even compared to effects of changes in mean environmental conditions, we need better projections of future climate change and its potential impacts on natural systems (Easterling et al. 2000; Meehl et al. 2000; Christensen and Hewitson 2007; Min et al. 2011). Moreover, climate change can cause fluctuations in other environment variables, such as soil nutrient availability which may be driven by rainfall patterns. As a consequence, increased climate and environmental variability will likely affect organisms in many different ways, and they need more attention from researchers.

How plants respond to environmental change

Terrestrial plants are among the most abundant living organisms on Earth. Ongoing global environmental change is challenging them by altering not only their regional climatic conditions, but also many other habitat factors such as resource availability, pollution and biotic interactions. The sessile lifestyle of plants limits their ability to escape from such unfavoured conditions, and therefore their phenotypic plasticity and ability to adapt *in* situ play important roles.

Plants are generally known to possess high phenotypic plasticity, i.e. the ability of a single genotype to produce different phenotypes depending on environmental conditions. This may involve changes in a range of ecologically important traits, from morphology, development, life-history to cross-generational effects (Sultan 2000). For instance, resource allocation – an important class of plant traits reflecting how a plant invests resource to different organs to mediate its growth (Poorter and Nagel 2000) – has been found to be plastic in many species. In the annual buckwheat *Polygonum persicaria*, low light availability triggers higher biomass allocation to leaf tissue, which in turn increases photosynthetic tissue (Sultan 2003), whereas low nutrient availability triggers higher allocation to root tissue to improve access to this limited resource (Sultan 2003). Several previous studies showed that plastic responses can improve average fitness across environments and are therefore adaptive (van Kleunen and Fischer 2005; Palacio-López et al. 2015).

Plastic responses can not only be induced by current environments, but also by the environmental conditions of parental organisms and thus be expressed across generations - a phenomenon known as "transgenerational plasticity" (Roach and Wulff 1987; Agrawal 2001; Donohue 2009). Just as within-generation plasticity, transgenerational plasticity can be adaptive (Galloway and Etterson 2007; Herman and Sultan 2011). For example, in *Arabidopsis thaliana* the progeny of heat- and salt-stressed plants exhibits enhanced stress tolerance, suggesting adaptation to ancestral environments (Whittle et al. 2009; Boyko et al. 2010). Transgenerational plasticity can be considered a special case of phenotypic plasticity, where responses are longer-term and require some kind of transfer of information from the parental environment.

While phenotypic plasticity is one possibility how plants can adjust their development to maintain fitness in changing environments, another possibility is that they adapt to changing environments through evolutionary changes. Plant populations can adapt to changing environments by altering their genetic composition (Reznick and Ghalambor 2001). If there is spatially variable natural selection, and heritable variation in relevant phenotypic traits, evolution will result in rapid adaptation to altered local conditions (Williams 1966; Kawecki and Ebert 2004). Since recent climate change poses strong selection pressure, adaptive evolution to changing climates can be very rapid. For example, Franks et al. (2007) demonstrated rapid evolution of flowering time in the annual plant *Brassica rapa* in response to an extreme drought in just a few generations. Thompson and co-workers (2013) found evolution of genetically-controlled chemotypes in response to reduced winter freezing through altered chemotype composition of populations of the Mediterranean plant *Thymus vulgaris*. In a recent review of the field, Franks et al. (2014) found 35 published studies with some evidence of rapid evolutionary responses of terrestrial plants to global climate change.

In natural communities, there is plenty of evidence that global change affects plant community composition, diversity and productivity, particularly with respect to elevated temperature and elevated CO_2 (Parton et al. 1995; Walther et al. 2002; Soussana and Lüscher

2007). These responses in productivity and community composition are consistently recorded across different ecosystems, from temperate grasslands, forests to high-latitudinal tundras (Rustad et al. 2001; Zavaleta et al. 2003; Walker et al. 2006; Wu et al. 2011), and models predict that even more profound long-term community changes are to be expected as climate change continues (Epstein et al. 2000).

Changes in population and community composition and diversity generally reflect the responses of individual genotypes and species (Callaway and Walker 1997; Tylianakis et al. 2008; Bolnick et al. 2011), and their interactions. Thus, in order to understand population- and community-level changes in response to global change, we first need to understand the variation in responses among different genotypes and species, respectively.

Variation among and within species

Different species differ in their growth forms, life histories, as well as morphological and functional traits. Some of these differences are easily observed, e.g. woody plants have a different growth form and life span than herbaceous plants, while grasses with shallow roots differ from forbs that more often have a deep root system. There are many other important, and less easily visible, ecologically important plant traits, such as growth rate, leaf life-span, leaf N:P ratios and biomass allocation, which can vary 10- to 100-fold among species, and in their responses to different environments (Grime and Hunt 1975; Reich et al. 1992; Güsewell 2004; Poorter et al. 2012). Interspecific trait variation can reflect long-term adaptation, for instance differences in the plasticity of congeneric *Polygonum* species responding to resource availability correspond to their contrasting environmental distributions (Bell and Sultan 1999; Sultan 2003). Species differences also reflect their evolutionary history. Already Darwin (1859) stated that differences among species also reflect evolutionary history, and that more closely related species are more likely to share functional and ecological similarities. More recently, researchers started to account for the phylogenetic relatedness between species to correct for non-independence in comparative studies of traits (Felsenstein 1985; Harvey and Pagel 1991). The degree of phylogenetic determination of traits is described by the so-called *phylogenetic signal* which quantifies the association of phylogeny with trait values across species, and thus to what extent there is evolutionary trait conservation (Blomberg and Garland 2002). Phylogenetic signal is now commonly considered when studying patterns of species-level trait variation (Blomberg et al. 2003), and there is for instance evidence of a phylogenetic signal in plant responses to climate change (Davis et al. 2010).

Just as species differences in environmental responses determine community/diversity changes (Walther 2010), the other important level of variation in natural communities is the variation within species, which determines how genetic diversity and composition will change at the population-level. Genotypes of the same species often differ in their traits and their responses to environmental change (Hughes et al. 2008; Lepš et al. 2011; Jung et al. 2014). The genetic variation in phenotypic traits within populations is the raw material for evolution by natural selection (Fisher 1930), often resulting in local adaptation. At the same time, the standing genetic variation within populations provide potential for future adaptation (Hedrick et al. 1976; Hedrick 1986; Kawecki and Ebert 2004). For instance, in the model plant *Arabidopsis thaliana*, researchers have detected large amounts of natural genetic variation in a wide range of habitats, and have linked this natural phenotypic variation to their source environments (Koornneef et al. 2004; Stinchcombe et al. 2004; Fournier-Level et al. 2011). Many

studies have demonstrated genetic variation among natural *A. thaliana* accessions e.g. in their responses to different light environments (Maloof et al. 2001; Botto and Smith 2002). A recent study found that genotype-specific adaptation to parental temperature in *A. thaliana* is related to climate of origin, suggesting adaptation to local climatic conditions (Groot et al. 2017).

Plant responses to environmental variability: what we know and what we don't know

While a large body of research exists on plant responses to global environmental change in general (e.g. Melillo et al. 1993; Walther et al. 2002), research focusing on increased environmental variability has not been so common, even though ecologists have repeatedly stressed the significance of environmental variability for many ecological processes such as species interactions and ecosystem functioning (Seastedt and Knapp 1993; Parmesan et al. 2000; Chesson et al. 2004; Knapp et al. 2008), and the need to distinguish between changes in environmental means and variability (e.g. frequency, magnitude, duration) in studies (e.g. Jentsch et al. 2007). Model simulations of current and future extreme weather patterns including increasing rainfall variability suggest that these will influence carbon fluxes and associated processes in terrestrial ecosystems (Medvigy et al. 2010). Indeed, empirical studies with experimentally altered rainfall patterns have demonstrated a wide range of responses in temperate grassland ecosystems, including changes in phenology, community structure, species diversity and ecosystem functions (Fay et al. 2000, 2003; Knapp et al. 2002; Heisler-White et al. 2008; Heisler-White et al. 2009), with some even finding that changing rainfall variability has stronger ecosystem effects than changing rainfall means. Also studies found that ecosystem responses strongly depended on the type of ecosystem investigated. For example, Heisler-White et al. (2009) showed that redistributing the same total amount of rainfall into large infrequent rainfall events caused aboveground NPP to increase in semi-arid and mixed grass prairies, whereas in mesic tallgrass prairies these effects were reversed. Furthermore, it has been shown that changing resource supply patterns could also lead to changes in community composition and affect the invasiveness of plants (Parepa et al. 2013). Although these community- and ecosystem-level studies inevitably raise questions about underlying species differences, there are so far only few studies that systematically compared environmental variability responses across multiple species. Those that did found interspecific variation both in the direction and magnitude of species responses to resource variability (e.g. Bilbrough and Caldwell 1997; Novoplansky and Goldberg 2001; Liu and van Kleunen 2017). Even less common are studies of within-species variation. I am aware of only two previous studies that tested for intraspecific variation in plant growth responses to fluctuating resource patterns (Poorter and Lambers 1986; Sher et al. 2004).

While a range of previous studies shed light on how increasing environmental variability can affect plant species and community dynamics, there are still many "unknowns", including, but not limited to (1) the effects of environmental variability *per se* and how they depend on other interacting processes, e.g. changes in environmental means, the presence of competitors, etc, (2) the relative importance of different components of environmental variability, such as the timing, frequency, duration and intensity of temporal fluctuations, (3) the phenotypic plasticity of plants in response to increasing environmental variability, and whether it is adaptive and can be transgenerational, and (4) how much inter- and intraspecific variation exists in such plant responses, and which ecological and

evolutionary factors explain this variation. Clearly, there is a great demand for carefully designed experiments that are able to separate effects of environmental variability from environmental means, and that systematically investigate the plant responses across multiple species and/or genotypes.

The goals of this thesis

The goal of my thesis was to address some of the research gaps outlined above, and to improve our understanding of how plants respond to increasing environmental variability. I use short-lived plants as study system, because short-lived species are likely to be more sensitive to interannual and seasonal climate variability than longer-lived species which are better able to buffer environmental fluctuations over longer time periods (Xia et al. 2010; Cleland et al. 2013). Another advantage of short-lived plants is that it is relatively easy to assess their fitness. I carried out a series of controlled experiments in which I manipulated environmental variability *per se*, sometimes in combination with environmental means, to be able to directly compare their effects. In all of my experiments, I focused on either among- or within-species variation in responses to increasing environmental variability, or both, and I tried to connect this variation to its ecological and evolutionary relevance.

In chapters II & III I present two greenhouse experiments that investigated the responses of annual plants to nutrient fluctuations, using a broad range of species that are common and ecologically relevant in Central Europe. Specifically, in **chapter II** I used multiple geographic origins of multiple annual weeds to assess both inter- and intraspecific variation in plant responses to nutrient variability, and I did this with a three-way full factorial experiment with changing overall nutrient means, increased nutrient variability and competition. **Chapter III** then is a much more thorough examination of interspecific variation using 37 different annual species, and I used this power of many test species to also test for a phylogenetic signal in plant responses to nutrient fluctuations.

In chapters IV & V I present a second set of two linked experiments which focused on the response of *Arabidopsis thaliana* to temperature fluctuations, and which on the one hand attempted to disentangle the timing and frequency components of variability, and on the other hand tested for transgenerational effects of the temperature fluctuations. The experiments took place in growth chambers where the timing and frequency of temperature stress could be precisely controlled. In both experiments I also examine natural intraspecific variation and its relationship with climate of origin. Specifically, **chapter IV** investigated the plastic responses of a range of natural *A. thaliana* ecotypes to temperature variability, with changes in the timing and frequency of temperature stress, and **chapter V** then assessed the transgenerational consequences of these responses for offspring, and the adaptive significance of the transgenerational effects. In both chapters I linked the variation among *A. thaliana* ecotypes back to the climatic variability of their origins.

Chapter II

Inter- and intraspecific variation in response to nutrient fluctuations in annual plants

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Abstract

As a consequence of global change, plants may experience increased temporal variability in soil nutrients. To predict the potential impact of nutrient fluctuations on plant populations and communities, we need to understand the extent of inter- and intraspecific variation in plant responses to such changes. To address these questions, we experimentally subjected multiple genotypes of 11 common annual plant species to different levels of temporal nutrient variability, at low and high nutrient levels, and with or without the presence of an interspecific competitor. We found that while changes in nutrient variability had generally weaker effects than competition or changes in nutrient means, increased nutrient variability had positive effects on the growth of some species, but had no or even negative effects on others. In five of the studied species we also found that different genotypes of the same species responded differently to increased nutrient variability. Thus, there is both inter- and intraspecific variation in how annual plants respond to nutrient fluctuations, and we therefore predict that increased nutrient variability alone – even if total nutrients do not change at all – will eventually alter the genetic and species composition of annual plant communities.

Keywords

annual plants, competition, genetic diversity, intraspecific variation, nutrient variability, species turnover

Introduction

The abiotic environment is changing worldwide (IPCC 2014), and biological organisms are responding. They shift their phenologies (Walther et al. 2002; Menzel et al. 2003; Badeck et al. 2004; Parmesan 2006; Ge et al. 2015) and spatial distributions (Walther et al. 2002; Moiseev and Shiyatov 2003; Parmesan 2006), or adapt to the novel conditions through evolutionary changes (Bradshaw and Holzapfel 2006; Merilä 2012). The extent to which species can respond depends on their biology, in particular their phenotypic plasticity, mobility and evolutionary potential. When the speed and magnitude of species responses is insufficient, species decline and may go extinct (Lenoir and Svenning 2015). Globally, there is evidence of an ongoing decline in population numbers and a strong overall loss of biodiversity (Ceballos et al. 2017).

Environmental change comes in two forms: changes in the means of environmental factors, or changes in their temporal variability. There is strong evidence that industrialization and land use (Crowley 2000; Folland et al. 2001; IPCC 2013) resulted in important changes in mean environmental factors such as warming or increased nitrogen deposition (Crowley 2000; Folland et al. 2001; IPCC 2013; IPCC 2014), and that the consequences of these changes spread across levels of biological organization, from individuals and populations to communities, ecosystems and biomes (Olesen and Bindi 2002; Salazar et al. 2007). At the same time, environmental variability has been changing as well (Easterling et al. 2000; Luterbacher et al. 2004; Schär et al. 2004; Dai 2012), and models predict that in many areas the temporal variability of environments will further increase in the future. Although it is likely that these changes also impact natural ecosystems (Easterling et al. 2000; Asseng et al. 2011), the ecological and evolutionary effects of changes in variability are still little understood (Post and Stenseth 1999; Walther et al. 2002).

The most important drivers of global environmental change are land use, climate and nitrogen deposition (Sala et al. 2000), and plants are directly affected by all of them (Foley et al 2005; Allen et al. 2010; Simkin et al. 2016). Interactions between drivers can further increase their impacts. For example, temperature and precipitation changes affect the mean availability and composition of soil resources (Vitousek 1994; Swift et al. 1998; Conant et al. 2001; Rustad et al. 2001; Guo and Gifford 2002; Jones et al. 2005), and both land use changes and climate extremes such as severe drought and intense rainfall events can create strong disturbances and episodes of increased nutrient availability (Swift et al. 1998; Sánchez et al. 2004), thus increasing temporal variability.

We know that plants react to environmental changes, and that their responses can be measured at multiple levels. When the environment changes, plant individuals can be plastic and display different phenotypes better suited for the new conditions (Chevin et al. 2010; Oostra et al. 2018). In addition, environmental change also exerts natural selection (Merilä 2012; Kingsolver and Buckley 2017), and given sufficient standing variation, plant populations can evolve and adapt, with better suited genotypes becoming more abundant over time. If some species are better able to adjust to environmental change than others, this will inevitably change ecological communities along with the functions and services they provide (Nelson et al. 2013). On even longer timescales, environmental change may drive species-level adaptation and speciation (Peischl and Kirkpatrick 2012), and some existing species differences likely reflect different environmental conditions experienced in the past. Thus, to understand population- and species-level adaptation, and to predict future community changes, it is important to quantify both within- and among-species differences in responses to environmental change.

Until now, the evidence for rapid evolutionary responses of plants to environmental change mostly comes from studies on climate change (Franks et al. 2014), whereas we know much less about rapid evolution in response to nutrient changes. Some previous studies investigated adaptation to increased nutrient means and found that there is local adaptation in responses to nitrogen availability (Gahoonia and Nielsen 1997; Treseder and Vitousek 2001; Zhu et al. 2005a, 2005b; Vergeer et al. 2008). We know particularly little about how plants respond to temporal fluctuations in nutrient availability. A few previous studies explored this question and showed that plant species differ in their responses to nutrient fluctuations (Crick and Grime 1987; Benner and Bazzaz 1988; Campbell and Grime 1989; Miao and Bazzaz 1990; Bilbrough and Caldwell 1997; Liu and van Kleunen 2017), and that nutrient fluctuations can influence plant invasion and thus community composition (Parepa et al. 2013). To our knowledge, only one previous study explored intraspecific variation in response to nutrient fluctuations: Poorter and Lambers (1986) experimented with two inbred lines of *Plantago major* and found that increased frequency of fluctuations favoured one of the lines over the other.

Here, we tested for both inter- and intraspecific variation in responses to nutrient fluctuations within and among 11 annual plant species. We used a full-factorial design and tested responses to increased nutrient variability, at low and high overall nutrient levels, and with or without the presence of an interspecific competitor. Specifically, we addressed the following questions: (1) What are the overall effects of increased nutrient variability on the growth of the studied plants, how large are these effects compared to the (well-studied) effects of changes in nutrient levels, and do they depend on nutrient levels and the presence of competitors? (2) How much variation is there among species in their responses to nutrient variability? (3) Do the studied plant species harbour significant intraspecific variation in responses to nutrient variability?

Material and methods

Experimental design

To test the questions outlined above, we conducted a greenhouse experiment in which we subjected 11 annual plant species to a factorial combination of nutrient level (low/high), nutrient variability (low/high) and competition (with/without) (Fig. 1). The 11 studied species were: *Bromus hordeaceus*, *Centaurea cyanus*, *Crepis capillaris*, *Galium album*, *Geranium robertianum*, *Lapsana communis*, *Myosotis arvensis*, *Papaver rhoeas*, *Sinapis arvensis*, *Trifolium arvense* and *Viola arvensis*. In the remainder of this paper, we refer to them by the genus name only. All seeds came from a specialized producer of wild seed material (Rieger-Hoffmann GmbH, Blaufelden-Raboldshausen, Germany) who produces seeds for ecological restoration from seeds collected in natural populations across Germany. For each species the seeds from different regions are collected and produced separately, which allows to study intraspecific variation and adaptation. Molecular and phenotypic studies have shown that the seeds maintain a substantial fraction of natural diversity and adaptation (Bucharova et al. 2017; Durka et al. 2017). The number of regions from which seeds are produced differs between species, and we purchased all regional ecotypes available, which resulted in a total of 48 distinct geographic origins for the 11 plant species.

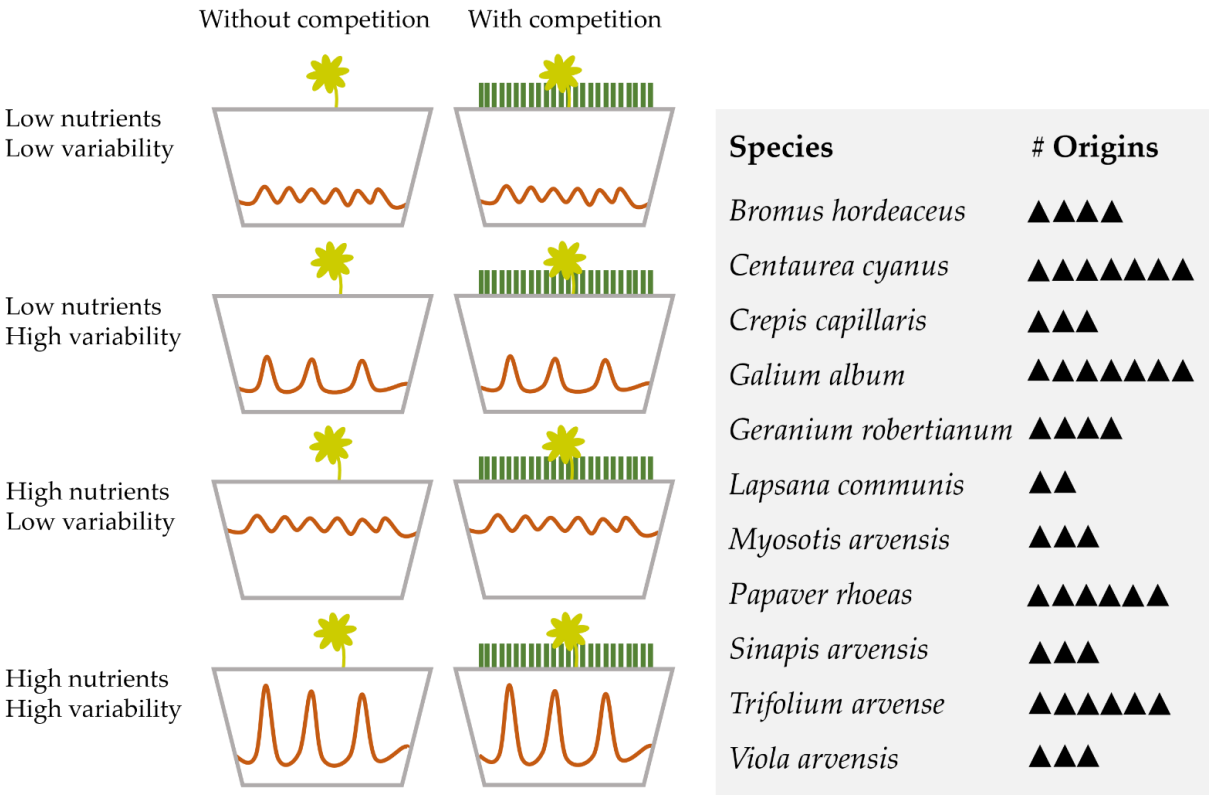

Figure 1. Schematic of the experiment, with a full-factorial combination of nutrient level (low/high), nutrient variability (low/high) and competition (with/without), and a total of 11 annual plant species and 2-7 different geographic origins (indicated by black triangles) subjected to each of these treatment combinations.

We germinated all seeds in July 2017 and transplanted the germinated seedlings into 7×7×8 cm pots filled with a 1:2 mixture of local soil and sand (Sand- und Kieswerk Bischoff, Rottenburg). Where possible, we transplanted several seedlings into a pot and thinned them down to one after successful establishment. The experimental treatments started in September and lasted for 50 days, with 10 nutrient applications at 5-day intervals. We used liquid fertilizer to create a low-nutrient treatment where the plants received a total amount of 2 g N m^{-2} during the experiment, and a high-nutrient treatment where they received a total of 6 g N m^{-2}. In the low-variability treatment, identical amounts of nutrients were applied at each of the ten time-points, whereas in the high-variability treatment, the plants received nutrients only at every second time point. To avoid a confounding of nutrient treatments with water availability, the high-variability plants received equal volumes of water without fertilizer at the other five time-points. Finally, to test how plant responses to different nutrient conditions depended on the presence of competitors, we grew all plants with or without adding 0.75 mL seeds of the annual grass *Poa annua* to the pots. For each of the eight treatment combinations (2 nutrient levels × 2 levels of variability × 2 competition scenarios), we planted five replicates per plant origin. For a few origins we had fewer than five replicates, so we ended up with 1820 planted seedlings. The pots were placed in a greenhouse in a completely randomized order, and were re-randomized monthly during the experiment. Some mortality (establishment failure, early senescence, herbivory) further reduced plant numbers during the experiment, so that eventually only 1666 plants were included in the data analyses.

Data collection

To document variation in initial plant sizes, we measured either plant height or the length of the longest leaf, depending on the species, one week before the start of the nutrient treatments. During the experiment we continuously recorded plant phenology as the number of days from germination until the first flowering of a plant. One week after the nutrient treatments had stopped, we harvested all the plants. From pots without competitors, we carefully extracted and rinsed the roots and separated the plant biomass into above- and belowground parts, whereas from pots with competitors, we only harvested the aboveground biomass of the focal plant and the competitor. All biomass samples were then dried at 60 °C for 72 hours and weighed. In addition to the aboveground biomass and flowering time data, which we had collected for all plants, we calculated the root:shoot ratio (belowground biomass divided by aboveground biomass) of plants grown without competitors, and the competitive success (aboveground biomass of the target plant divided by the total aboveground biomass in a pot, i.e. sum of target plant and competitor) of plants grown with competitors.

Data analysis

For the data analyses, we had four dependent variables: (1) flowering time and (2) aboveground biomass, which could both be analysed for all plants, (3) root:shoot ratio, which was available only for plants without competitors, and (4) competitive success, which was available only for plants with competitors. Flowering time could be analysed for only two of the species, *Centaurea* and *Sinapis*, because these were the only species where sufficient flowering occurred during the experiment (in 98 *Centaurea* plants and 71 *Sinapis* plants). The data for aboveground biomass, root:shoot ratio and competitive success were square-root transformed before the data analyses. To correct for initial variation in plant sizes prior to the main analyses, we first fitted a linear model with only initial size as explanatory variable, and then used the residuals from these models for all subsequent analyses.

To get an idea of the overall effects of our experimental treatments, we first ran a mixed model on all flowering time and aboveground biomass data that included nutrient level, nutrient variability, and competition, and all of their interactions, as fixed factors, and another one on all root:shoot ratio and competitive success data that included only nutrient level, nutrient variability and their interaction. Species as well as origins nested within species were included as random factors in these cross-species analyses. Next, we tested for intraspecific variation by including plant origins, and their interactions with the experimental treatments, as fixed factors into the models. Because of unequal sample sizes and numbers of origins for the different species, and to avoid excessive post-hoc testing, we ran these analyses separately for each species. The models were thus either three- or four-factorial, depending on the response variable, and included nutrient level, nutrient variability, competition (only for flowering time and aboveground biomass) and plant origin, and all possible interactions. All analyses were done in R (R Core Team 2017).

Results

Overall effects of nutrient variability

Overall, nutrient level, nutrient variability and competition all significantly affected plant growth. On average, competition decreased aboveground biomass by 80%, and nutrient levels increased biomass by 55% (Fig. 2A) but also accelerated flowering and affected root:shoot ratio and competitive success (Table 1). Compared to the effects of competition and nutrient level, the effects of increased nutrient variability were rather moderate. Across all plants, increased nutrient variability caused a significant increase of aboveground biomass by 4.65% (main effect of nutrient variability in Table 1, Fig. 2A). In addition, there was a significant three-way interaction between nutrient variability, nutrient level and competition for plant aboveground biomass (Table 1), where nutrient variability had a positive effect ($P = 0.001$) on biomass in the absence of competitors and at high nutrient levels, but not in the presence of competitors or at low nutrient levels (Fig. 2B). We found no effects of increased nutrient variability on the root:shoot ratio or competitive success of the studied plants (Table 1).

Species differences

The effects of increased nutrient levels and competition were very consistent across the 11 studied plant species. In nine out of the 11 species increased nutrient levels had a positive effect on aboveground biomass (Table 3, Fig. S1), and in nine species it decreased root:shoot ratio (Table 4, Fig. S1), i.e. where nutrients were abundant (and competitors absent) plants invested less into nutrient acquisition structures. Nutrient levels affected competitive success in five of the 11 species (Table 4); in four of these competitive success decreased, and only in one species it increased at high nutrient levels (Fig. S1). Finally, competition strongly decreased aboveground biomass in all 11 species (Table 3). Of course, while the direction of these effects were consistent, their magnitudes differed among species.

In contrast to the effects of nutrient levels and competition, plant responses to nutrient variability were not only weaker but also more variable across species. In *Centaurea*, the effect of nutrient variability on flowering time depended on the presence of competitors, with accelerated flowering in response to increased nutrient variability when competitors were present, but delayed flowering when competitors were absent. In *Sinapis* there was a significant three-way interaction, with accelerated flowering in response to increased nutrient variability only at high nutrient levels and in the presence of competitors (Table 2). Increased nutrient variability had a positive effect on aboveground biomass of *Crepis*, *Galium*, *Lapsana*, *Papaver* and *Trifolium*, whereas it had a negative effect on that of *Sinapis*, and no significant effect on the other five species (Table 3, Fig. 3). There were also species differences in how the root:shoot ratio (Fig. 4A) and competitive success (Fig. 4B) of plants was affected by increased nutrient variability. In two species, *Crepis* and *Galium*, root:shoot ratio increased significantly in response to increased variability, whereas in most other species it had little or no effect (Table 4). Increased nutrient variability had a significant positive effect on the competitive success of *Trifolium*, but it had little effects on the competitive success of the other species.

Table 1. Results of linear mixed models testing for the overall effects of nutrient level, nutrient variability, competition, and their interactions, on aboveground biomass, root:shoot ratio and competitive success (all 11 species). The values are *F*-values, with significant effects indicated by colour shading: $P < 0.05$, $P < 0.01$, $P < 0.001$.

	Aboveground biomass	Root:shoot ratio	Competitive success
Nutrient level (NL)	271.1	124.9	23.3
Nutrient variability (NV)	4.7	1.2	0.8
Competition (C)	4440.1		
NL × NV	0.4	0.5	0.8
NL × C	89.2		
NV × C	3.2		
NL × NV × C	4.3		

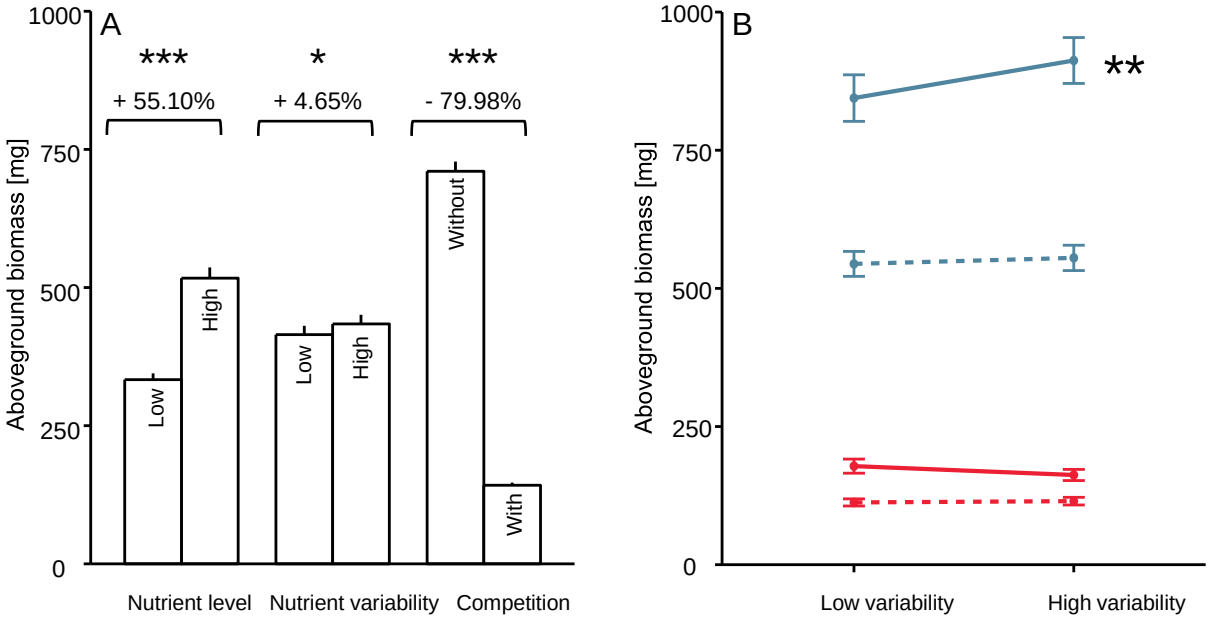

Figure 2. Overall responses of aboveground biomass across all 11 studied plant species. (A) Main effects of nutrient level, nutrient variability and competition, with significant changes indicated by asterisks, and average % change given for each main effect. (B) Responses of aboveground biomass to changes in nutrient variability, depending on nutrient levels and the presence of competitors. Plants without competitors are in blue, plants with competitors in red; solid lines indicate high nutrient levels, dashed lines low nutrient levels. Significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Intraspecific variation

There were significant origin effects (main effects of origin or origin by treatment interactions in Tables 2-4), confirming the presence of intraspecific variation, in all of the studied species. The magnitudes of these effects differed among traits and species, from rather weak origin effects, e.g. in *Centaurea* and *Lapsana*, to much stronger effects, e.g. in *Geranium* and *Trifolium*. In several of the species there were significant origin by nutrient level or origin by competition interactions, indicating genetic variation in nutrient plasticity and competitive ability, respectively. Moreover, there were significant origin by nutrient variability interactions for aboveground biomass in *Galium*, *Geranium* and *Sinapis*, for root:shoot ratio in *Galium*, *Myosotis* and *Sinapis*, and for competitive success in *Galium*, indicating that these species were also genetically variable in their responses to nutrient variability (effects of origin by nutrient variability interactions in Tables 3-4, Figures 3-4).

Table 2. Results of linear models testing for the flowering time responses of individual species to the experimental treatments, and for intraspecific variation in these responses. The values are *F*-values, with significant effects indicated by colour shading: $P < 0.05$, $P < 0.01$, $P < 0.001$. The first row indicates the number of geographic origins and total number of plants included per species.

	Centaurea	*Sinapis*
# origins / # samples	7 / 93	3 / 68
Nutrient level (NL)	13.7	1.6
Nutrient variability (NV)	0.0	4.0
Competition (C)	9.6	0.0
NL × NV	2.8	3.7
NL × C	2.6	0.2
NV × C	5.7	1.0
NL × NV × C	0.3	4.6
Origin (O)	1.3	20.0
O × NL	1.6	1.3
O × NV	0.4	0.6
O × C	0.4	0.1
O × NL × NV	0.9	0.1
O × NL × C	0.8	2.8
O × NV × C	1.8	1.3
O × NL × NV × C	1.3	

Table 3. Results of linear models testing for the aboveground biomass responses of individual species to the experimental treatments, and for intraspecific variation in these responses. The values are *F*-values. Significant effects are indicated by colour shading: □ $P < 0.05$, □ $P < 0.01$, ■ $P < 0.001$. The first row indicates the number of geographic origins and total number of plants included per species.

	Bromus	*Centaurea*	*Crepis*	*Galium*	*Geranium*	*Lapsana*	*Myosotis*	*Papaver*	*Sinapis*	*Trifolium*	*Viola*
# origins / # samples	4 / 157	7 / 229	3 / 116	7 / 238	4 / 125	2 / 55	3 / 115	6 / 195	3 / 68	6 / 234	3 / 93
Nutrient level (NL)	181.6	99.9	33.6	27.6	65.6	24.3	34.7	100.4	4.5	1.1	0.1
Nutrient variability (NV)	0.3	0.0	4.5	13.0	0.0	4.5	0.7	4.3	6.3	4.8	2.0
Competition (C)	755.4	1181.8	832.0	981.2	1782.2	283.8	297.6	629.8	58.0	1353.7	283.9
NL × NV	1.7	3.1	0.2	2.4	0.0	1.4	1.7	0.4	0.3	1.6	0.9
NL × C	16.1	42.9	19.2	9.9	58.2	11.0	4.5	26.6	8.9	1.1	0.2
NV × C	1.0	0.6	2.1	3.7	4.2	0.0	0.8	0.0	0.0	0.1	0.2
NL × NV × C	0.2	0.9	0.0	0.0	2.2	0.2	2.0	2.2	0.9	1.3	0.5
Origin (O)	13.8	1.6	4.0	8.5	6.7	0.5	2.6	3.5	2.7	33.6	8.6
O × NL	1.3	1.2	0.9	0.5	1.1	3.9	1.6	0.5	0.7	0.7	8.0
O × NV	0.2	1.4	1.3	3.0	3.6	0.0	0.2	2.1	6.7	0.7	1.3
O × C	5.4	0.5	3.1	5.9	12.0	0.6	6.9	12.8	2.4	23.8	1.3
O × NL × NV	0.6	1.3	1.2	0.4	5.9	0.4	0.8	1.1	1.8	1.7	0.0
O × NL × C	2.5	3.1	1.1	0.9	1.6	0.5	0.2	1.0	1.8	0.7	4.2
O × NV × C	0.6	0.8	0.2	2.0	3.0	0.8	3.1	2.5	1.0	1.2	1.4
O × NL × NV × C	1.4	0.8	0.4	0.8	6.9	1.0	3.8	0.5	0.7	1.6	0.1

Table 4. Results of linear models testing for the individual-species responses in root:shoot ratio and competitive success to the experimental treatments, and for intraspecific variation in these responses. The values are *F*-values. Significant effects are indicated by colour shading: □ $P < 0.05$, □ $P < 0.01$, ■ $P < 0.001$. The first row indicates the number of geographic origins and total number of plants included per species.

	Bromus	*Centaurea*	*Crepis*	*Galium*	*Geranium*	*Lapsana*	*Myosotis*	*Papaver*	*Sinapis*	*Trifolium*	*Viola*
Root:shoot ratio											
# origins / # samples	4 / 79	6 / 92	3 / 51	6 / 115	4 / 77	2 / 29	3 / 56	6 / 95	3 / 34	6 / 116	2 / 40
Nutrient level (NL)	39.9	37.1	34.0	8.7	28.2	0.3	20.8	9.9	10.5	0.2	31.3
Nutrient variability (NV)	0.8	1.8	4.9	6.1	0.0	1.7	0.5	0.0	1.5	2.3	0.5
NL × NV	0.2	3.2	0.0	4.2	1.4	5.3	2.6	0.2	0.9	0.2	0.0
Origin (O)	4.8	3.6	3.5	5.8	4.1	0.4	11.7	1.5	1.3	2.2	0.3
O × NL	0.3	1.7	1.0	2.3	2.2	22.6	0.2	1.3	0.7	2.4	0.1
O × NV	0.4	1.1	1.2	3.1	0.6	0.4	5.3	1.7	4.7	2.1	1.2
O × NL × NV	0.2	0.7	0.6	0.7	1.1	0.1	3.7	0.5	0.7	0.2	1.8
Competitive success											
# origins / # samples	4 / 78	7 / 136	3 / 59	7 / 122	4 / 48	2 / 26	3 / 58	6 / 89	3 / 34	6 / 117	3 / 53
Nutrient level (NL)	0.3	2.9	3.1	4.5	0.2	2.0	0.8	7.0	9.2	74.7	6.6
Nutrient variability (NV)	4.0	1.8	0.8	0.4	0.3	1.4	1.6	2.0	0.0	7.5	0.0
NL × NV	1.1	0.9	0.4	1.8	1.2	1.6	2.0	0.0	1.1	0.1	0.2
Origin (O)	4.1	1.4	1.0	1.9	8.0	0.3	2.4	26.4	4.0	9.2	25.3
O × NL	2.4	2.7	2.7	0.8	0.3	0.5	1.2	0.4	0.4	1.5	0.0
O × NV	0.3	1.9	1.1	3.1	0.4	0.0	1.0	1.9	2.8	0.9	1.0
O × NL × NV	0.3	1.1	2.4	1.5	1.9	0.5	1.1	1.0	0.0	0.9	0.0

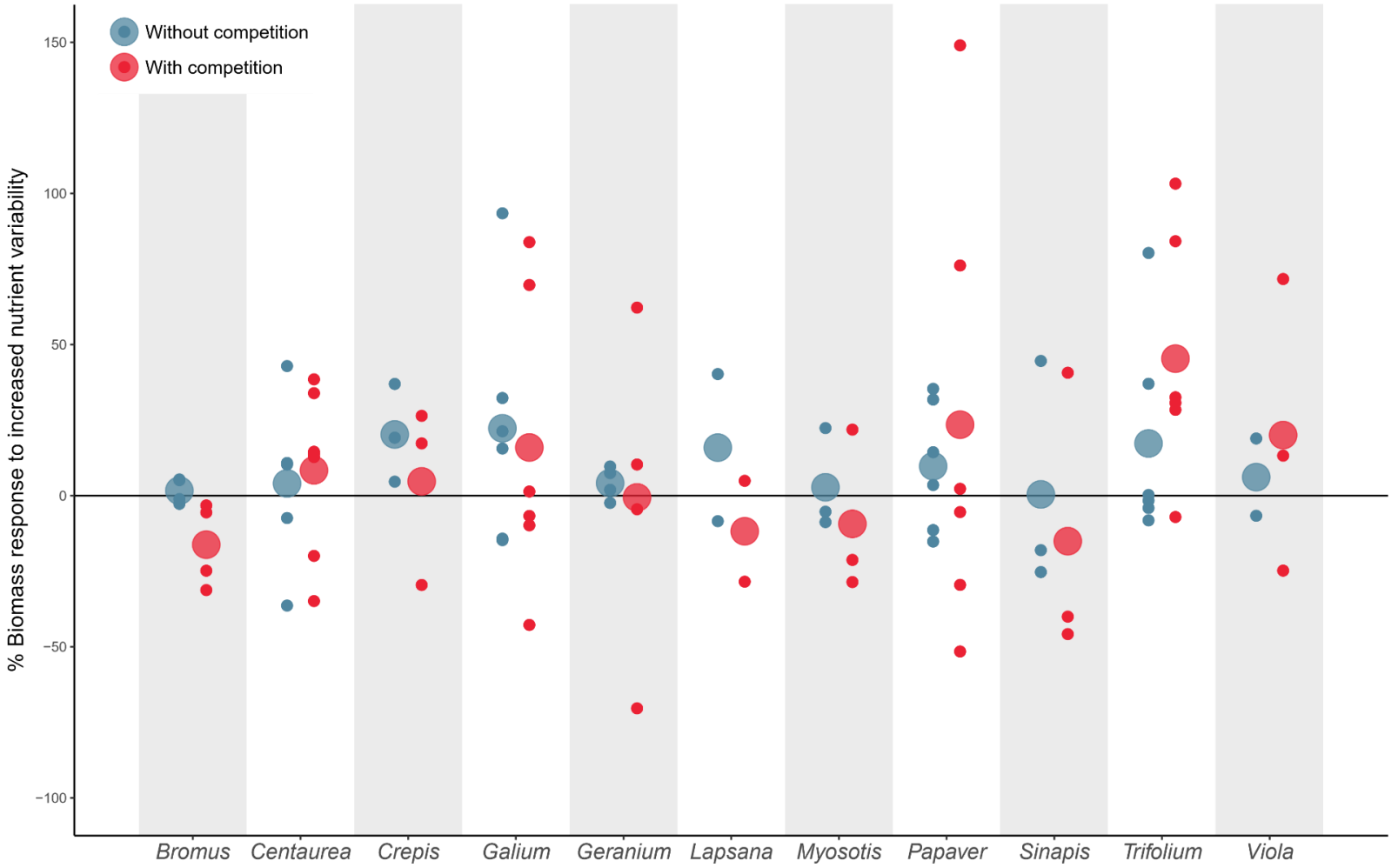

Figure 3. Responses of plant aboveground biomass to increased nutrient variability, separately for each of the 11 studied annual plant species, and for plants grown without (blue) and with (red) competition. The values are the % changes in response to increased nutrient variability. The large bubbles are the species means, and the smaller dots are the average responses of different geographic origins within a species.

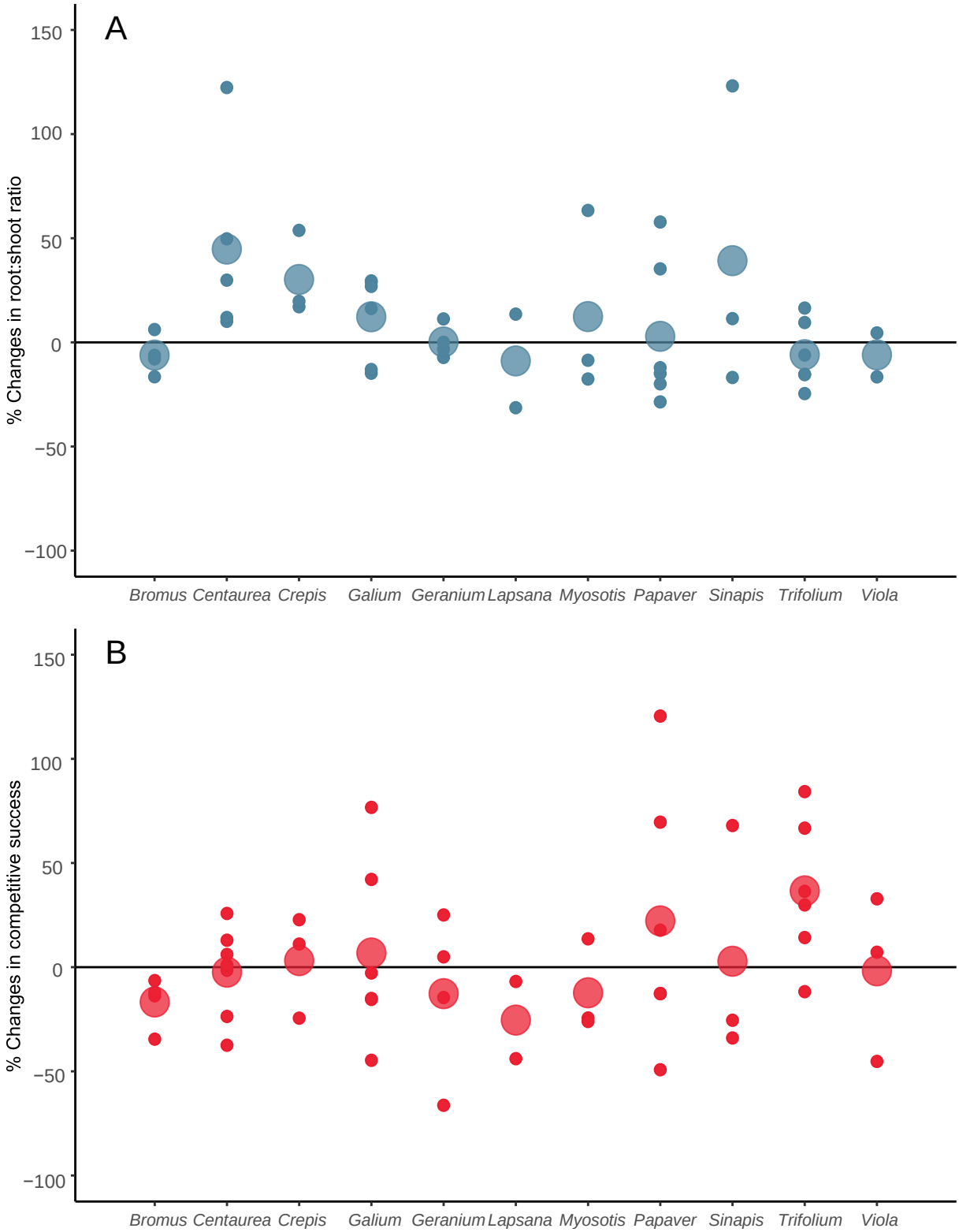

Figure 4. Responses of (A) root:shoot ratio and (B) competitive success to increased nutrient variability, separately for each of the 11 studied annual plant species. The values are relative changes calculated as (average at high variability – average at low variability) / average at low variability. The large bubbles are the species means, and the smaller dots are the average responses of different geographic origins within a species.

Discussion

If plant species or genotypes differ in their responses to environmental changes, then these will alter plant communities and change their genetic composition. From a plant's perspective, one of the key environmental factors are soil nutrients, and we already know a lot about the ecological and evolutionary responses of plants to increased nutrient levels. Here, we find that changes in the temporal variability of nutrients alone, without any changes in their total amounts, can affect plant growth, and that species and genotypes differ in their responses to nutrient variability. Our results indicate that changes in temporal variability of nutrient conditions can significantly affect the diversity and evolution of plant communities.

Overall effects of nutrient variability

We found that across all 11 studied species, increased nutrient variability significantly affected plant biomass, but that compared to the strong effects of nutrient level and competition the effects of nutrient variability were rather moderate. Increased nutrient variability had a positive effect on studied plants, but only in the absence of competitors and at high nutrient levels. The competitor we used was the grass *Poa annua*. Previous studies found that nitrogen addition favors grasses over forbs and legumes (Stevens et al. 2006; Wragg 2017), and our results indicate that *Poa annua* is indeed a strong competitor in nutrient-rich environments but also under more variable nutrient conditions, so the species must be better able to take advantage of temporary nutrient surpluses. In the absence of *Poa annua*, however, the target plants benefitted from increased nutrient variability and significantly increased their biomass. This is particularly intriguing since total nutrient levels did not change. A possible explanation could be that plants compete for nutrients also with soil microorganisms (Jackson et al. 1989; Hodge et al. 2000; Inselsbacher et al. 2010; Kuzyakov and Xu 2013), and changes in temporal nutrient variability may have affected the competitive balance between microbes and plants. Soil microorganisms are known to take up nutrients faster than plants during the first 1-2 days after fertilization (Jackson et al. 1989; Inselsbacher et al. 2010), but after that plant uptake becomes more efficient, so that plants can have an edge over microorganisms (Jaeger et al. 1999; Inselsbacher et al. 2010). Moreover, the nutrient uptake of soil microorganisms is thought to be more efficient at low nutrient concentrations (Kuzyakov and Xu 2013). If this is true, then small frequent nutrient pulses may favour microorganisms in maintaining a higher uptake rate and relative amount of captured nutrients, whereas with larger and less frequent nutrient pulses, where microorganisms have reached their uptake limits, plants main regain strength. A more variable nutrient environment could therefore shift the competitive balance between plants and microbes towards the plants. Future studies could test this idea by manipulating soil microbes or explicitly analysing their activity in nutrient fluctuation experiments.

Another intuitive explanation for the success of the studied plants under temporally variable, nutrient-rich conditions could be that all of them are common in grasslands, ruderal places and/or agricultural fields – habitats that are often nutrient-rich and regularly disturbed (= reduced competition) or fertilized (= nutrient pulses). Thus, one may be tempted to think that they may possess a superior nutrient-use efficiency to utilize nutrient pulses. However, some previous studies found that, on the contrary, species from nutrient-poor habitats were better able to exploit temporary nutrient pulses compared to species from nutrient-rich soils

(Crick and Grime 1987; Campbell and Grime 1989), so this is at least not a universal explanation.

Species differences

We found that plant responses to increased nutrient variability differed among the 11 tested species. Five species produced more biomass under fluctuating nutrient conditions, whereas five others did not respond, and one (*Sinapis*) grew even less well. There was less interspecific variation with regard to the other traits, but this probably also reflected the reduced data sets and smaller overall effect sizes of nutrient variability in these analyses.

Part of the differences in species performance may be related to their varying growth stage and therefore developmental demand and uptake capacity for nutrient pulses. Plant growth stage has been shown to affect nutrient responses (Benner and Bazzaz 1988; Bilbrough and Caldwell 1997), and plants in earlier developmental stages are likely to have greater plasticity. In our study, *Trifolium* was the the slowest-growing species, and it was the only one where competitive success increased with increased nutrient variability. This observation also agrees with a previous study showing that increased resource heterogeneity favours slow-growing plants in interspecific competition (Novoplansky and Goldberg 2001). In the two fastest-growing species (*Centaurea* and *Sinapis*), increased nutrient variability had an effect only under stressful environments (with competition), where it induced earlier flowering, and thus possibly an 'escape strategy' of speeding up the life cycle to avoid unfavorable periods (Franks et al. 2007). Similar effects have been found by other studies, e.g. Fay et al. (2000) who showed that increased rainfall variability shortened flowering time in a mesic grassland. The only species in our experiment that produced significantly *less* biomass at higher nutrient variability was *Sinapis*, the species with the fastest life cycle and therefore, presumably, the one with the least flexible development at the time when the treatment started.

Together with a handful of previous studies (Crick and Grime 1987; Benner and Bazzaz 1988; Campbell and Grime 1989; Miao and Bazzaz 1990; Bilbrough and Caldwell 1997; Liu and van Kleunen 2017), our results show that some species are better able to exploit large infrequent nutrient pulses than others. This has important community-level implications: if increased nutrient variability favours particular species, then natural or anthropogenic changes in the temporal patterns of nutrient supply will inevitably change plant community composition and diversity, just as changes in nutrient levels are known to do (Socher et al. 2013; Wragg 2017). Community-level tests of this hypothesis are so far scarce, and restricted to short-term mesocosm experiments, but they find significant community changes in response to increased temporal variability (Parepa et al. 2013). Longer-term studies on the effects of increased environmental variability so far only exist for water availability, and they even found that changes in temporal water variability have stronger effects on community diversity and ecosystem functioning than changes in the total amount of water (Fay et al. 2000; Knapp et al. 2002). Clearly, further studies, and particularly more natural and more long-term ones, are needed to confirm these predictions also for nutrient variability.

Having established the presence of interspecific variation, an important next question is which traits explain the observed variation among species. Our results for the other traits provide some clues: Two of the species with the strongest positive biomass response to increased variability, *Crepis* and *Galium*, were also the only ones where nutrient variability had a significant main effect on the root:shoot ratio. The root:shoot ratio of these two species

increased in response to nutrient variability, indicating that an enlarged root system, and more generally plasticity in allocation patterns, may be beneficial traits in a more temporally variable environment. This is consistent with previous studies which also found increased root allocation to be advantageous for plants that experience large, unpredictable nutrient pulses (Crick and Grime 1987; Campbell and Grime 1989).

Intraspecific variation

To test for intraspecific variation, our experiment included multiple geographic origins of each of the 11 studied species. We found that the geographic origins differed significantly in each species, confirming the presence of natural variation in the wild seed materials used (see also Bucharova et al. 2017; Durka et al. 2017). However, the strength of the origin effects differed greatly among species, and these differences did not simply result from the variable number of orgins tested, as some species with only few origins, such as *Geranium*, showed strong differentiation whereas others with larger sample sizes, such as *Centaurea*, were little differentiated.

Most importantly, several species also harboured significant intraspecific variation in their responses to increased nutrient variability, with the strongest and/or most consistent intraspecific variation in *Galium*, *Geranium*, *Myosotis* and *Sinapis*, whereas other species did not. Our study thus confirms that response to nutrient variability can be a genetically variable trait, just as other types of plasticities, including plant response to nutrient levels (e.g. Pigliucci et al. 1995; Pigliucci and Schlichting 1995; Cahill et al. 2005). Interestingly, the observed intraspecific variation in response to nutrient variability seems to have little to do with variation in response to nutrient levels, since many of the species in our study harboured only one of the two. For instance, *Sinapis* origins differed in their response to nutrient variability but not nutrient levels, whereas for *Viola* the opposite was the case. This indicates that the two types of responses likely have a different genetic and functional basis.

So far, only very few studies have tested for heritable within-species variation in plant responses to environmental variability *per se*. Poorter and Lambers (1986) examined genotype differences in responses to changing nutrient frequencies, and Sher et al. (2004) demonstrated differences between Mediterranean and desert origins in responses to temporal water fluctuations). Clearly more studies are needed, but the evidence so far has several implications: First, the existence of intraspecific variation means that plant response to nutrient variability is an evolvable trait, and if standing genetic variation exists within natural populations, then natural or anthropogenic changes in temporal nutrient variability will lead to shifts in the genetic composition of these populations (Barrett and Schluter 2008). Second, we found intraspecific variation in response to nutrient variability only in some of the studied species, which suggests that some species, such as *Galium*, *Geranium* and *Sinapis*, will be better able to adapt to increasingly variable nutrient conditions than others. Finally, if the observed intraspecific variation is adaptive, then differences between seed origins may reflect differences in nutrient variability of the source habitats. More detailed studies, in particular combinations of field studies with common gardens, and possibly experimental evolution approaches will be needed to test these ideas and predictions.

Conclusions

Our study shows that changes in the temporal variability of nutrient availability alone can impact the growth of plants, but the degree to which this happens depends on the plant species and genotype, i.e. there is both inter- and intraspecific variation in this trait. Since global change involves also changes in environmental variability, including changes in the temporal patterns of nutrient conditions, it is important to understand the biology of nutrient variability responses, and their ecological and evolutionary implications. Future studies should try to elucidate the functional mechanisms – traits and their genetic basis – underlying the observed species and genotype differences, and they should in particular attempt to test the predictions generated from this study through long-term community and evolution experiments.

Acknowledgements

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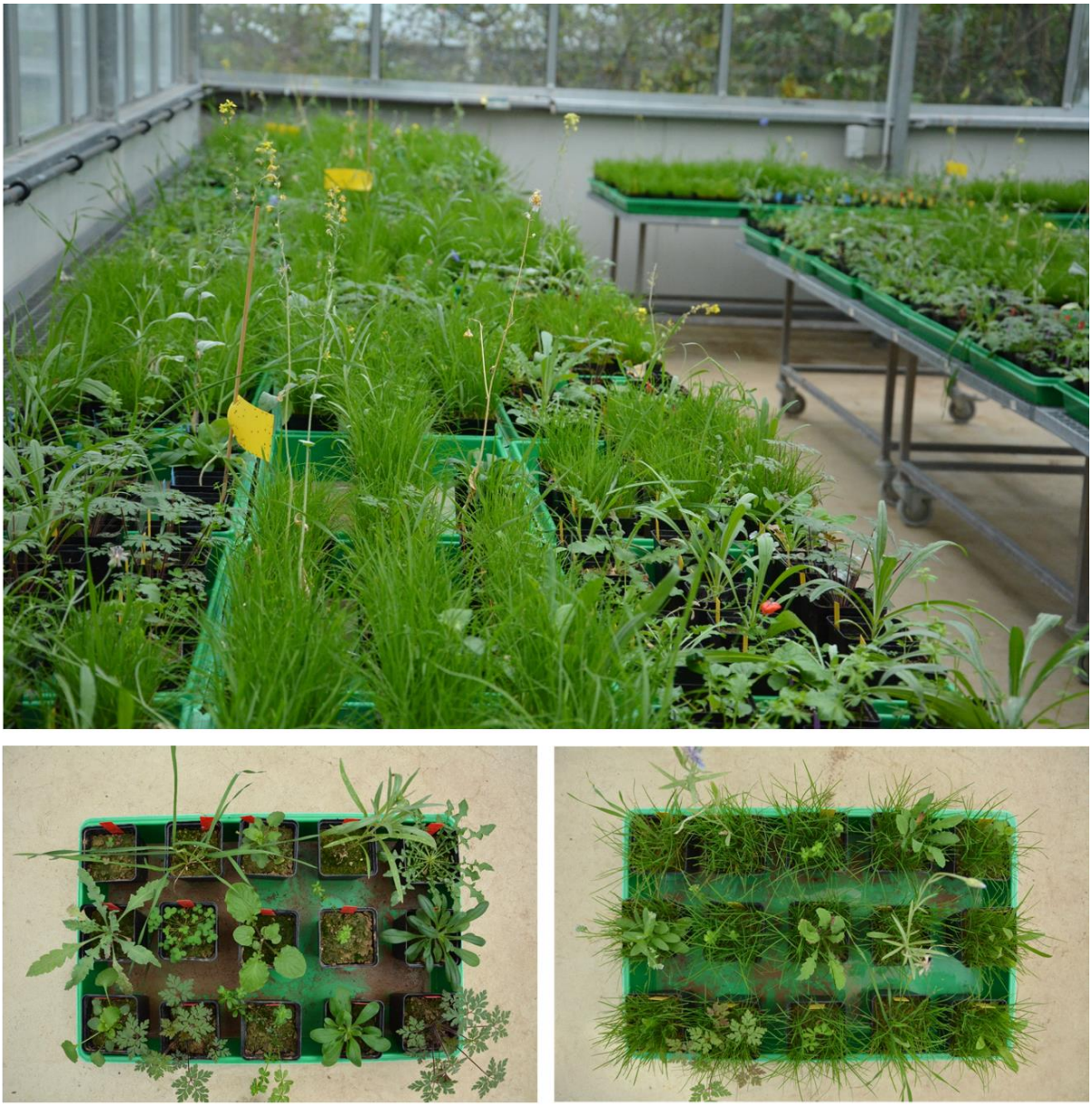

Impression from the greenhouse experiment (top), and plants growing without (left) versus with (right) the competitor *Poa annua*. (Photo: Y Deng)

Supplementary information

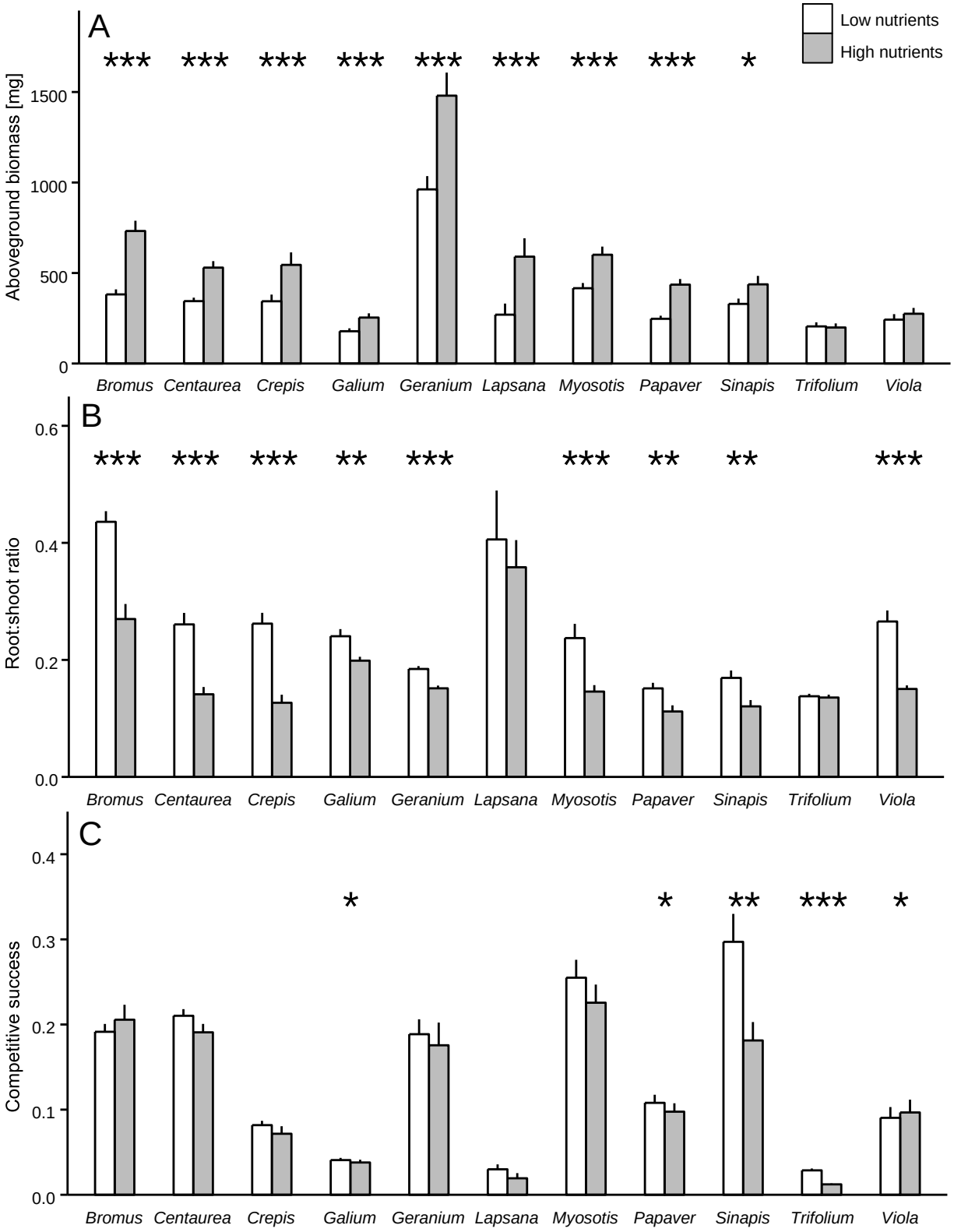

Figure S1. Responses of (A) aboveground biomass, (B) root:shoot ratio and (C) competitive success to changes in nutrient level, separately for each of the 11 studied annual plant species. Significant effects of nutrient level are indicated by asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Chapter III

Species differences and phylogenetic signal in response to nutrient fluctuations among 37 annual plant species

Ying Deng, J.F. Scheepens, Oliver Bossdorf, Madalin Parepa

Abstract

To predict global change effects on ecological communities, we need to understand how species differ in their responses to changing environments. Some variation among species can be explained by their evolutionary relatedness, and there is thus a recent trend of studying phylogenetic patterns in global change responses. One aspect of global change that has so far received relatively little attention is the increasing temporal variability of environmental conditions, including nutrient fluctuations which can be caused, among others, by increased climatic variability or land use changes. To gain insight into species-level variation in plant responses to increased nutrient variability, we conducted a multi-species experiment with 37 common annual plants subjected to a combination of different nutrient levels, increased nutrient variability and competition. We found substantial species variation, as well as a significant phylogenetic signal, in plant responses to all three treatments, indicating that plant responses to competition, nutrient levels and nutrient variability are all long-term evolving and phylogenetically conserved traits. Most importantly, species-level variation in responses to nutrient variability was uncorrelated to species responses to nutrient level or competition. Thus, plant response to nutrient variability appears to be a distinct species trait, and changes in the temporal patterns of nutrient availability will therefore also have distinct effects on the diversity and composition of natural plant communities.

Keywords

competition, environmental variability, functional traits, interspecific variation, nutrient pulses

Introduction

The study of plant responses to global environmental changes has been a major theme in ecological research of the last decades. Many previous studies have investigated how environmental change affects the phenotypes or distribution of individual species, as well as the diversity and invasibility of plant communities (e.g. Dukes and Mooney 1999; Bakkenes et al. 2002; Cleland et al. 2007). To understand global change effects on communities we need to have an idea of the response differences among species (Parmesan and Yohe 2003) because different responses of co-occurring species will inevitably result in changes of plant community structure and diversity, as observed in many ecosystems (Zavaleta et al. 2003; Walker et al. 2006; Walther 2010). It is therefore important to conduct comparative studies of environmental responses across multiple species in order to predict how communities will shift under changing environmental conditions.

Among-species variation in responses to environmental change can be linked to both the ecology and evolution of species. In particular, since all species are hierarchically linked through their evolutionary history (Mayr 1982), the variation in many species characteristics can often be partly explained by phylogeny, and the application of phylogenetically informed comparative analyses has therefore become a common tool for understanding ecological differences between species (Felsenstein 1985; Harvey and Pagel 1991). For example, biomass allocation patterns differ between eudicots and monocots (Poorter et al. 2012), and angiosperms generally differ from gymnosperms in their seed size (Moles et al. 2005). The degree of phylogenetic association can vary from trait to trait due to, among others, differences in physiological constraints or in trait sensitivities to environmental selection. For instance, life history traits such as flowering time are generally more labile and usually less phylogenetically conserved than body size or other morphological traits (Blomberg et al. 2003; Davies et al. 2013).

An important metric for linking phylogeny and trait variation is the so-called *phylogenetic signal*, which describes the strength of phylogenetic determination of a particular trait (Blomberg and Garland 2002). Several measures for phylogenetic signal have been developed, such as Abouheif's C_{mean} (Abouheif 1999), Pagel's λ (Pagel 1999), Moran's I (Gittleman and Kot 1990) or Blomberg's K (Blomberg et al. 2003). All have in common that they compare the observed traits against some null models where species are randomized across the phylogenetic tree, for instance the Brownian motion model (Martins 1996) which assumes trait variation to increase proportionally with evolutionary time. The use of phylogenetic signal is not only useful for detecting evolutionary trait conservatism in multi-species studies (Blomberg et al. 2003; Davies et al. 2013), but it can also be of value in understanding species responses to environmental changes (e.g. Davis et al. 2010).

Environmental change can be changes in the means of environmental factors, but also in their temporal variability (Easterling et al. 2000; Meehl and Tebaldi 2004; Min et al. 2011; Rummukainen 2012). Most previous research on plant responses to environmental change was concerned with how changes in the mean temperature, CO_2, precipitation or other environmental factors impact plant individuals, populations and communities (e.g. Rustad et al. 2001; Root et al. 2003; Ainsworth and Long 2005; Menzel et al. 2006; Wu et al. 2011). However, in addition to the well-known trends of shifting environmental means, there have also been observations of increasing temporal variability, for instance increased intensity and/or frequency of warming and precipitation events, at regional and continental scales (Groisman et al. 1999; Luterbacher et al. 2004; Meehl and Tebaldi 2004; Schär et al. 2004; Dore

2005; Goswami et al. 2006). Such climatic variability changes can also impact natural ecosystems (Easterling et al. 2000), and alter the phenology, geographic distribution and community dynamics of plants (White et al. 1997; Knapp et al. 2008; Jackson et al. 2009).

Besides climate, another major driver of plant community structure and dynamics in terrestrial ecosystems is nutrient availability. Climate change and other anthropogenic processes can alter the spatial and temporal distribution of plant-available nutrients. For instance, increased climate variability should also increase variation in soil nutrients, because nutrient availability in the soil is known to be affected by temperature as well as soil moisture (Chapin et al. 1995; Knapp et al. 2008). However, while the effects of increased nutrient levels, caused by land use and atmospheric deposition, on plant diversity and ecosystem functioning have been well-studied (e.g. Gough et al. 2000; Stevens et al. 2004; Suding et al. 2005; Bobbink et al. 2010), we so far know little about the ecological consequences of increased nutrient variability. Several previous studies tested effects of rainfall variability on grassland ecosystems and found strong impacts on productivity and species diversity (e.g. Knapp et al. 2002; Fay et al. 2000, 2003; Heisler-White et al. 2008; Heisler-White et al. 2009). For instance, Knapp et al. (2002) showed that experimentally increased rainfall variability promoted species richness and evenness of a mesic grassland, and that this effect was related to variability in soil moisture. However, since soil nutrients are generally only plant-available when dissolved in water (Cassman and Munns 1980), the variability in soil moisture must inevitably have been accomponied by variability in soil nutrients, and thus some of the ecosystem effects observed by Knapp et al. (2002) may have been driven by soil nutrient variability. One previous study that directly tested the effects of nutrient variability on experimental plant communities found that increased temporal nutrient variability promoted exotic plant invasion into these communities (Parepa et al. 2013).

As for other environmental changes, examining plant responses to nutrient fluctuations across many different species will help to establish general patterns (van Kleunen et al. 2014), and to understand the potential community-level consequences of increased nutrient variability. So far only few studies examined plant responses to nutrient fluctuations across multiple species, and these have mostly been limited to small numbers of species (e.g. Crick and Grime 1987; Benner and Bazzaz 1988; Campbell and Grime 1989; Miao and Bazzaz 1990; Bilbrough and Caldwell 1997), with one exception: Liu and van Kleunen (2017) examined the responses of 29 native and non-native herbaceous plant species to different temporal patterns of nutrient supply and found that on average the non-native species responded more positively to strong nutrient pulses.

Under natural conditions, changes in total availability of resources and in their temporal patterns often happen simultaneously, and their effects on plants may be interactive. For instance, Fay et al. (2000) found that increased water variability had stronger effects on plant phenology at reduced overall levels of water availability. Moreover, uptake of resources is affected by the presence of neighbours. For instance, in a semi-arid community Clarke et al. (2005) found that the effects of rainfall seasonability on native plant abundance strongly depended on the presence of buffel grass, demonstrating that interspecific competition altered the ecological effects of resource fluctuations. Therefore, studying plant responses to resource variability under different overall resource levels, and with or without competitors, will give us a more complete, and more realistic, picture.

The goal of our study was to investigate plant responses to nutrient variability across a broad range of 37 plant species, under different nutrient and competition levels, and to test for phylogenetic signals in these responses. We thus asked the following three main questions:

(1) Do plant species differ in their responses to nutrient fluctuations? (2) How are species responses to nutrient variability related to their responses to nutrient means and to competition? (3) Is there a phylogenetic signal in the studied plant responses?

Material and methods

In a greenhouse experiment, we subjected 37 European annual plant species (Fig. 1) to a full-factorial combination of nutrient levels (low/high), nutrient variability (low/high) and competition (with/without). In April 2018, we obtained seeds from commercial suppliers (Rieger-Hofmann GmbH, Blaufelden-Raboldshausen, Germany; Templiner Kräutergarten, Templin, Germany; B & T World Seeds, Aigues-Vives, France) and planted individual seedlings in 7×7×8 cm pots filled with a 1:2 mixture of local soil and sand (Sand- und Kieswerk Bischoff, Rottenburg). All plants were then subjected to a treatment period of 50 days, during which we supplied each pot with liquid fertilizer (N:P:K=7:5:6; toom GmbH, Cologne, Germany) distributed in ten rounds of applications of 5 mL at 5-day intervals. The total amount of fertilizer per pot was equivalent to 2 g N m^{-2} for the low-nutrient treatment and 6 g N m^{-2} for the high-nutrient treatment. Under the low-variability treatment, the same amount of nutrients was applied at each of the ten time-points, whereas under the high-variability treatment, the double amount of nutrients was applied at every second time point, and at the time points without nutrient applications the same volume of water (5 mL) was applied. Half of the pots from each of the four nutrient-treatment combinations were randomly assigned to the competition treatment and were sown with 0.75mL *Poa annua* seeds. Overall, we set up five replicates of each species for each of the eight treatment combinations, for a total of 1480 pots. Before the start of the treatments all pots were randomly distributed in a greenhouse, and then re-randomized once after a month. The plants were watered as needed, i.e. water was not a limiting factor throughout the experiment.

Data collection

One week before the start of the nutrient treatments, we measured the sizes of all seedlings. Depending on the species, we used plant height or the length of the longest leaf as measure of initial size. During the experiment, we continuously recorded flowering phenology as the number of days from germination until the first flower opening. 21 species flowered during the experiment. One week after the end of the nutrient treatments we harvested all plants. For plants without competitors we washed the roots and separated the above- and belowground biomass, while for plants grown with competitors we separated the aboveground biomass of the focal plants and the competitor but did not harvest the roots. All biomass samples were dried at 60 °C for 72 hours and weighed. Low germination of a few species and early senescence of some focal plants reduced the total number of harvested plants to 1462. Besides the data of flowering time and biomass, we also calculated the root:shoot ratio (belowground biomass divided by aboveground biomass) for plants without competition, and the competitive success (biomass of focal plant divided by the sum of biomass from both focal plant and competitors) for plants with competition.

Data analysis

Our data analysis had three steps, corresponding to the three main study questions: First, we tested for interspecific variation in plant responses to the experimental treatments with linear models that included the main effects of nutrient level, nutrient variability, competition, and species, and all possible interactions between these factors. To account for variation in initial plant sizes, we first fitted a linear model with only initial size as explanatory variable and then used the residuals from these analyses for fitting the main model. We analysed four dependent variables: flowering time, aboveground biomass, root:shoot ratio and competitive success. For flowering time and aboveground biomass, we fitted the full four-factorial model, whereas for the root:shoot ratio data (pots without competition) and competitive success data (pots with competition) the variable competition was dropped and the model became a three-factorial. To improve normality of residuals, the aboveground biomass, root:shoot ratio and competitive success data were square-root transformed prior to the analyses. All analyses were done in R (R Core Team 2017).

Second, we tested for species-level correlations between plant responses to nutrient level, nutrient variability and competition. To be able to compare treatment effects across species, we first standardized all raw data, and then we fitted separate linear models for each species. For flowering time and aboveground biomass, we fitted a full three-factorial model with nutrient level, nutrient variability, competition and their interactions, and for root:shoot ratio and competitive success we used a two-factorial model with only nutrient level, nutrient variability and their interaction. From each individual-species model, we extracted the estimates of the main effects, and we then used these data to calculate species-level correlations between species responses to nutrient level, nutrient variability and competition.

Third, we used the same standardized effect sizes for treatment main effects to test for phylogenetic signals in species responses to the experimental treatments. We obtained a phylogenetic tree of our 37 species through *phylomatic* (Webb and Donoghue 2005). Then we used the R package *phylosignal* (Keck et al. 2016) to test for phylogenetic signals in response variation. According to Münkemüller et al. (2012), under Brownian motion model Abouheif's C_{mean} and Pagel's λ are considered to have better performance. We used Abouheif's C_{mean} to test for correlations between phylogeny and effect sizes (Abouheif 1999; Münkemüller et al. 2012; Keck et al. 2016), and we additionally calculated phylogenetic correlograms, based on Moran's *I*, to explore response autocorrelation (i.e. similarities) at different phylogenetic distances (Gittleman and Kot 1990; Keck et al. 2016). Finally, we attempted to identify 'hotspots' of phylogenetic signal (positive or negative autocorrelation), using so-called local indicators of phylogenetic association (LIPA; also based on Moran's *I*), which can indicate species with particularly similar or distinct trait patterns from their neighbours (Anselin 2010; Keck et al. 2016).

Results

Not surprisingly, nutrient level and competition generally had strong effects across species (Table 1). At high nutrient levels, the average aboveground biomass was by 84% higher, the root:shoot ratio was by 13% lower, and the competitive success was decreased by 9%. When grown with competitors, the target plants had on average a 63% lower biomass and flowered 3 days earlier. For nutrient variability, in contrast, there was no significant main effect, but its

effects depended on nutrient level and competition (NL × NV and NL × NV × C interactions in Table 1). High nutrient variability caused a decline of biomass production at high nutrient levels, but it had no effect at low nutrient levels (Fig. S1A). In contrast, the differences of competitive success increased under high nutrient variability, with plants being less competitive under high nutrients than under low nutrients (Fig. S1B). Furthermore, there was a three-way interaction among nutrient variability, nutrient level and competition in flowering time (NL × NV × C interaction in Table 1).

Table 1. Results of linear models testing the effects of nutrient level, nutrient variability, interspecific competition, species, and their interactions, in a greenhouse experiment with 37 annual plant species. The values are *F*-values, with significant effects indicated by colour shading: □ $P < 0.05$, □ $P < 0.01$, ■ $P < 0.001$.

	Flowering time	Aboveground biomass	Root:shoot ratio	Competitive success
Nutrient level (NL)	1.3	669.9	32.3	17.6
Nutrient variability (NV)	0.9	0.2	0.1	0.6
Competition (C)	49.4	2628.4		
NL × NV	4.4	5.2	1.3	10.5
NL × C	0.4	139.1		
NV × C	0.9	0.1		
NL × NV × C	4.3	3.0		
Species (S)	154.6	62.1	79.7	39.8
S × NL	1.2	3.6	2.0	1.8
S × NV	0.9	2.1	1.3	1.8
S × C	2.2	11.7		
S × NL × NV	1.1	0.7	0.7	1.4
S × NL × C	1.0	1.2		
S × NV × C	0.8	1.0		
S × NL × NV × C	2.1	1.4		

Variation in species responses

While we found overall effects of nutrient treatments and competition, their strength and direction depended on species identity. We found species variation in response to nutrient level for aboveground biomass, root:shoot ratio and competitive success, and there were species differences in response to competition in flowering time and aboveground biomass (S × NL and S × C interactions in Table 1). There was also species variation in plant response to

nutrient variability, particularly for aboveground biomass and competitive success (S × NV interactions in Table 1). Some species such as *Sinapis arvensis*, *Valerianella carinata* and *Alyssum alyssoides* were favored by increased nutrient variability and produced more biomass, whereas other species, such as *Viola arvensis* and *Sonchus asper*, responded negatively, and yet others such as *Agrostemma githago* and *Poa annua* were not affected (Fig. 1A). Finally, there was a four-way interaction between species, nutrient level, nutrient variability, and competition for flowering time, indicating that species variation in phenology responses was complex and depended on both nutrient level and the presence of competitors.

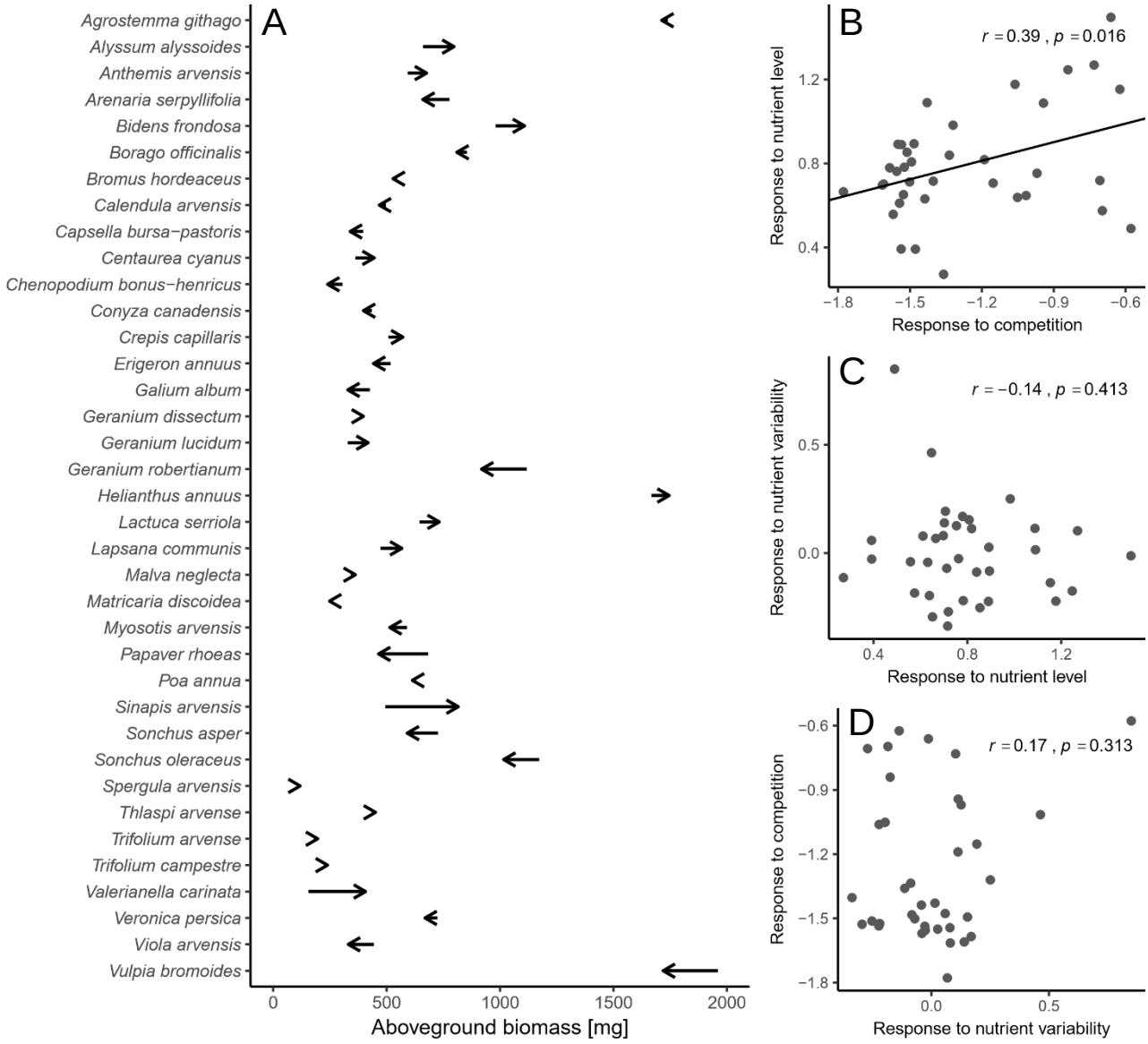

Figure 1. Responses of the 37 annual plant species to increased nutrient variability, with arrows indicating the changes in aboveground biomass from low variability to high variability (A), and species-level correlations between the biomass responses to the different treatments (B-D). Each dot represents one species. The values are parameter estimates for the main effects of the different treatments in separate linear models, using standardized data, for each species.

Correlation between species responses to treatments

In aboveground biomass, we found a significant positive correlation between species responses to nutrient level and to competition (R = 0.39, P = 0.016), but neither of them were correlated with species responses to nutrient variability (Table S1, Fig. 1B-D). In flowering time, root:shoot ratio and competitive success, we did not detect any significant correlation between species responses to different treatments (Table S1).

Phylogenetic signal in species response variation

We found a significant phylogenetic signal in the response of aboveground biomass to all three experimental treatments, while for root:shoot ratio the phylogenetic signal was weaker, and for competitive success and flowering time the signal was non-significant. In aboveground biomass, a phylogenetic signal was present in species responses to nutrient level (C_{mean} = 0.331, P-value = 0.003), nutrient variability (C_{mean} = 0.163, P-value = 0.037) and competition (C_{mean} = 0.242, P-value = 0.014) (Fig. 2), showing a strong influence of phylogeny on species variation in all three types of responses. The phylogenetic correlograms computed by Moran's I showed significant autocorrelation of species responses to nutrient level and competition at short phylogenetic distances, whereas for response to nutrient variability there was a similar, albeit not statistically significant, tendency (Fig. S2). The local Moran's I (LIPA) test detected significant signals in several species that had some degree of local similarity with their neighbours (Fig. 2). The response to nutrient level was particularly consistent in four families. *Caryophyllaceae* and *Poaceae* consistently showed strong responses of aboveground biomass to nutrient level, whereas *Fabaceae* and *Asteraceae* generally showed weak response (Fig. 2). Competition had a relatively weak effect on *Brassicaceae*, *Caryophyllaceae* and *Boraginaceae*, whereas it had the strongest effect on *Asteraceae*. In response to nutrient variability, only *Brassicaceae* showed significant local similarity and responded positively to increasing variability (Fig. 2).

In plants grown without competition, we also detected a significant phylogenetic signal in the response of root:shoot ratio (RSR) to nutrient variability (C_{mean} = 0.174, P-value = 0.041), but there was no phylogenetic signal in RSR responses to nutrient level (C_{mean} = - 0.235, P-value = 0.978) (Fig. S3). In spite of the overall lack of phylogenetic signal, the phylogenetic correlograms computed by Moran's I showed a significant negative autocorrelation at short phylogenetic distances and positive autocorrelation at larger distances (Fig. S4A) for RSR responses to nutrient levels, indicating dissimilarity in RSR responses in closely related species but similarity among more distantly related ones. For RSR responses to nutrient variability the phylogenetic correlograms did not detect any autocorrelation at all (Fig. S4B). In line with this, we found no local similarity 'hotspots' for RSR responses to nutrient level, and only very few in RSR responses to increased nutrient variability, where only in the phylogenetic neighbourhood of *Brassicaceae* there was a consistent clustering of positive RSR responses to increased nutrient variability (Fig. S3).

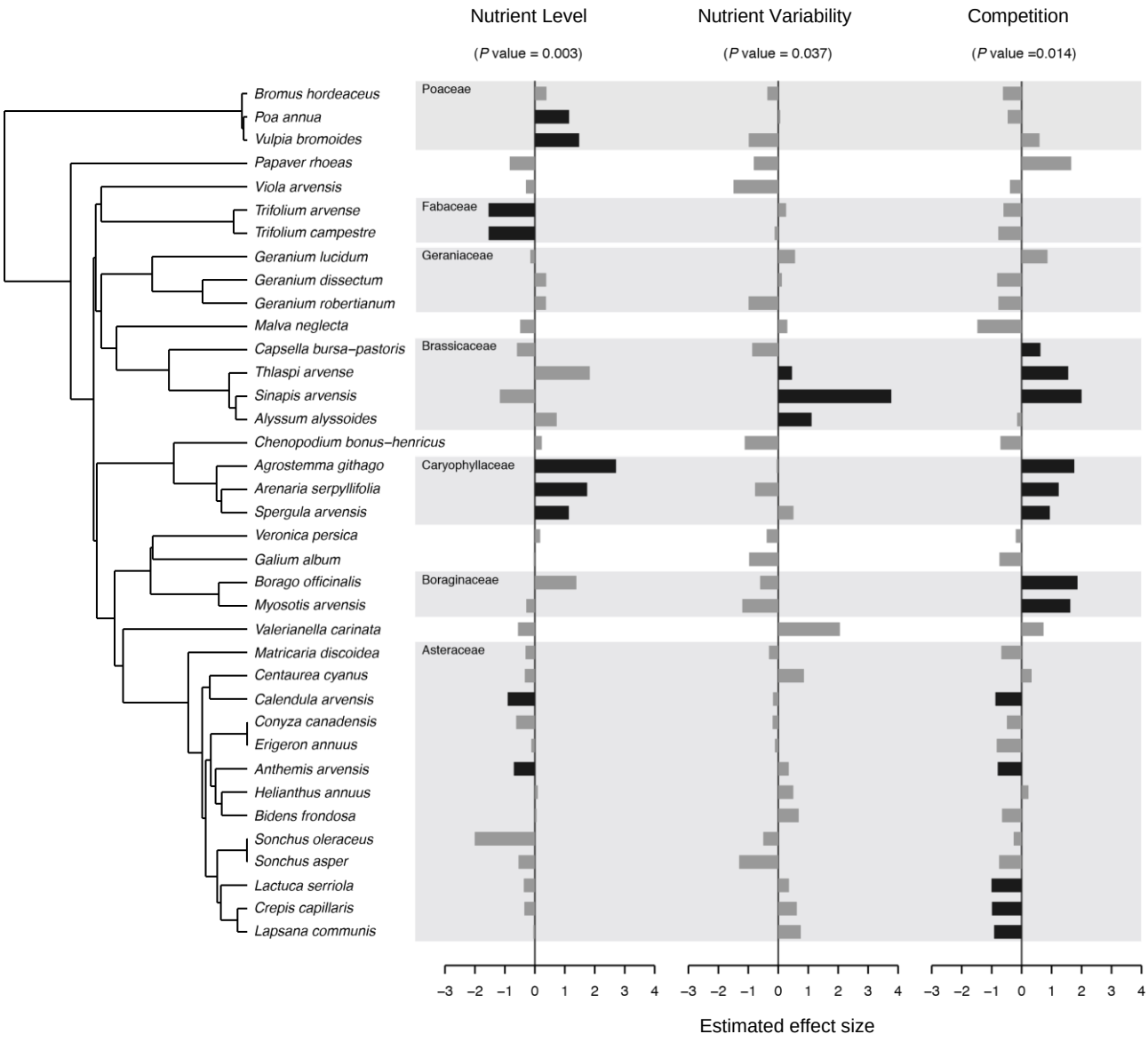

Figure 2. Response of aboveground biomass to nutrient level, nutrient variability and competition mapped on the phylogeny of the 37 studied species. The value are effect sizes of the respective main effects from individual-species models. The significance levels of the phylogenetic signal tests (Abouheif's C_{mean}) for each treatment are shown at the top. Dark bars are species with significant local Moran's *I* values, showing 'hotspots' of local positive autocorrelation.

Discussion

Increasing environmental variability is a dimension of global change for which the ecological impacts remain little understood. In this study we measured the response of 37 annual plants to increased temporal variability of nutrient supply, together with increasing mean supply and competition. We found that the response to nutrient variability does not correlate to that of changing nutrient means or of competition, and that for each type of response the phylogeny explains a significant amount of the variation.

In line with our expectations, the increase in nutrient level was beneficial for biomass production and decreased root allocation. This is consistent with the optimal partitioning theory which posits a higher allocation for plant organs that acquires the most limiting resource (Thornley 1972; Bloom et al. 1985), as found by several previous studies (e.g. Gedroc et al. 1996; Poorter and Nagel 2000; Liu and van Kleunen 2017).

In terms of temporal variability of nutrient supply, we found that variability alone does not strongly affect plant growth and phenology, but it could interact with both nutrient mean and inter-species competition. We observed that increased nutrient variability negatively affected productivity under high nutrients and resulted in reducing the biomass enhancement with increased total nutrients. Given that species in our study are mostly originating from nutrient rich habitats, this may suggest that they are likely to be sensitive to changes in nutrient supply and not favoured by increased disturbance. Thus an increased temporal variability of nutrients would suppress their growth. Previous observations on temporal water resource variability revealed that it could affect grassland productivity, and the effect differed depending on the water availability in a given grassland system. For instance, mesic grasslands with high water availability responded negatively to increased water variability, whereas semi-arid grasslands with low water availability were positively affected (Fay et al. 2000, 2003; Knapp et al. 2002; Heisler-White et al. 2009). Similar to this, our results suggest that in nutrient rich habitats, increasing nutrient supply variability may also affect community productivity by reducing the biomass productivity increase from nutrient enhancement (Gough et al. 2000; Zavaleta et al. 2003).

Previous studies from Fay et al. (2000) and Jentsch et al. (2009) reported that rainfall variability could drive shifts in plant phenology. In our study species the shift in flowering suggests that the phenological responses to resource variability are common in short-lived plants, and particularly in nutrient-rich environments and when there is strong competition. Increasing nutrient variability would then induce plants to flower early and shorten their life cycle and thus to escape from environmental fluctuations (Franks et al. 2007; Franks 2011). In addition, results from the competition treatment suggest that while the grass species *Poa annua* is a strong competitor in both nutrient-rich and nutrient-poor soil, it seems to be more successful in nutrient-rich and variable environments. While Zavaleta and co-workers (2003) found that nitrogen supply favours grass abundance in a grassland ecosystem, our results suggest that in such nutrient-rich communities increasing variability may also shift the inter-species competition balance towards grasses over other types of species e.g. forbs and legumes, and in the long run change their abundances in the community.

It is noteworthy that some plant phenological and physiological processes e.g. plant water relations, are especially vulnerable to climatic variability (Reyer et al. 2013). In our study the shifts of flowering phenology and the altered plant productivity indicate that phenological

and growth responses are potentially good predictors of the ecological consequences of environmental variability.

Species variation in response to nutrient fluctuations

Species varied in response to nutrient level, nutrient variability and competition. Previous studies on inter-species comparisons along nutrient gradients have revealed the differences in species plasticity to nutrients (e.g. Tilman and Wedin 1991; Wilson and Tilman 1995; McConnaughay and Coleman 1999). We have proved in our study that this is a common phenomenon in annual plants, and that the magnitude of species plasticity varies in terms of their productivity, biomass allocation, phenology as well as competitive ability. More importantly, we showed that species also greatly differed in their response to nutrient variability, a phenomenon much less recognized before. A few published studies have provided some early evidence of it (Crick and Grime 1987; Benner and Bazzaz 1988; Campbell and Grime 1989; Miao and Bazzaz 1990; Bilbrough and Caldwell 1997; Liu and van Kleunen 2017). Here we compared 37 annual species and our data shows strong evidence of species differences in response to environmental variability *per se*, in terms of both their direction and magnitude.

For plant communities, species variation in the response to nutrient fluctuations has important implications. If the difference that we measured in isolation or in competition to one other species holds within natural communities, then regardless of total overall nutrient availability, changes in temporal supply alone can influence species interactions and community composition. Our results show that increasing nutrient variability can promote some species (e.g. *Sinapis arvensis* and *Alyssum alyssoides*) whereas it can suppress others (e.g. *Violas arvensis* and *Sonchus asper*), and therefore may shift their relative abundance in communities. Similarly, there is evidence on how water resource variability changes community structure and species diversity (Knapp et al. 2002). Based on our results after testing 37 different species, we predict that nutrient variability has similar consequences. The mesocosm experiment of Parepa et al. (2013) revealed the potential impact of temporally varying nutrients in altering community composition. How well the prediction from single species and mesocosm responses holds for the community and ecosystem level needs to be further tested in more long-term and realistic systems.

Species responses to variability are independent from the responses to amount of nutrients or competition

In aboveground biomass, the positive correlation between the responses to nutrient level and to competition shows that plants that have greater response to available nutrients are usually more competitive. This corroborates observations from communities in which increased nutrient availability enhances species competition and increases the extinction risk of less competitive species, resulting in the loss of species diversity (Gough et al. 2000; Stevens et al. 2004; Suding et al. 2005). Moreover, what is intriguing in our study, is that species responses to nutrient variability were independent from that of nutrient level and competition. This is a novel finding showing that environmental variability acts independently from the environmental mean. Our study thus indicates that whilst changes in the resource amount shifts the competition between species, the variability in temporal supply patterns creates

another dimension of species variation. This may reduce the fitness differences between species induced by mean resource. It may therefore buffer competitive exclusion and maintain species co-existence and diversity (Chesson 2000; Knapp et al. 2002; Chesson et al. 2004).

Phylogeny explains species responses

Besides that species variation in response to environmental variability is not correlated with response to environmental mean, we also find that species phylogenetic relationships partly explain their response. Phylogenetic non-independence has been reported in a large array of species and it concerns their similarity in trait values and responses to environments (Freckleton et al. 2002; Blomberg et al. 2003; Davis et al. 2010; Davies et al. 2013). Our study is the first one to report its presence in species responses to both environmental mean and variability.

In our dataset, the strongest phylogenetic signal was found in biomass production. The decreased phylogenetic association in other traits such as phenology is likely due to the reduced sample size that caused lack of statistical power, or possibly the lability of such traits that are more tied to environmental cues rather than to evolutionary constraints, as found in other studies (Davies et al. 2013; Lessard-Therrien et al. 2014).

The correlation at short phylogenetic distance indicates a tendency of evolutionary conservatism in closely related species to respond similarly to environmental cues. Davies et al. (2013) argued that their finding on phenological trait conservatism may be attributed to genetically and geographically based conservatism, that closely related species are expected to have shared physiology and sensitivity to environmental cues (Harvey and Pagel 1991), and that generally species in this situation are expected to co-occur in similar habitats due to ecological reasons such as niche conservatism and environmental filtering (Webb 2000; Webb et al. 2002; Wiens and Graham 2005). In terms of conserved responses to nutrient fluctuations, the known genetic basis of nutrient uptake (Crawford 1995; Sunkar et al. 2007) and the physiological responses to environmental variability changes (Reyer et al. 2013) hint towards the hypothesis that the response to variability is genetically conserved, studies are needed to find out whether these genetic mechanisms are commonly shared by closely related species. At the same time observation on co-occurrence patterns in grassland communities provide mixed support (Silvertown et al. 1999; Silvertown et al. 2001), but there is evidence of environmental filtering on species co-occurrence which depends on both the spatial scale and the degree of environmental variation (Willis et al. 2010; de Bello et al. 2013). Furthermore, the weaker phylogenetic signal in response to nutrient variability than to nutrient mean and competition suggests that a reduced amount of variation is explained by phylogeny whereas an increased amount of variation is caused by other sources. Possible explanations for this could be (i) that there is more variance in plasticity in response to nutrient variability since we found that its effect was more variable, and (ii) that physiological processes more sensitive to changes in nutrient variability are evolutionarily more labile. Future insights into mechanistic understanding of plant responses to nutrient fluctuations could help to verify this speculation.

Our results suggest that some plant families conserve their response to nutrient variation. Under increased nutrients, *Caryophyllaceae* and *Poaceae* would likely benefit whereas *Fabaceae* and *Asteraceae* would have a relative disadvantage, and variable nutrients may benefit more the *Brassicaceae*. Given the small sample size in some families in our study, this result should be interpreted with care.

In the study from Davis et al. (2010) flowering time responses to seasonal temperature variation exhibited stronger phylogenetic signals than responses to century variation. Our results also proved that there is a phylogenetic pattern in species response to short-term variation in nutrient availability. This indicates that environmental variability at short time scales will elicit similar responses from closely related species and therefore phylogeny can be useful for understanding community changes under projected fluctuations in climate and resources. In the same way phylogeny was used to explain other ecological functions and processes such as host range of plant pathogens, community productivity, and plant invasions (Gilbert and Webb 2007; Cadotte, Cavender-Bares, et al. 2009; Cadotte, Hamilton, et al. 2009). While environmental filtering can be used to explain species assemblages (Alexander et al. 2011), community ecologists may also use phylogeny to help predict the changes in communities. Since phylogenetic association is expressed more strongly in competition (Burns and Strauss 2012), community experiments are likely to reveal stronger patterns of predicted responses, and are clearly needed to test these predictions.

Conclusions

Our study of nutrient fluctuations in 37 annual plant species suggests that short-lived plants respond differently to nutrient variability compared to mean nutrient availability and we provide evidence that the responses are phylogenetically related.

Acknowledgements

We thank Eva Schloter, Christiane Karasch-Wittmann, Yu-li Sui and Anna Kirschbaum for their help with the practical work. This work was supported through a CSC (China Scholarship Council) scholarship to YD.

Supplementary information

Table S1. Correlations between species responses to nutrient level, nutrient variability and competition in four plant traits. The *R*-values are Pearson correlation coefficients. Significant correlation ($P < 0.05$) is in bold.

	Flowering time		Aboveground biomass		Root:shoot ratio		Competitive success	
	R	*P*-value	*R*	*P*-value	*R*	*P*-value	*R*	*P*-value
Level vs Variability	0.14	0.593	-0.14	0.413	0.30	0.068	0.12	0.473
Variability vs Competition	-0.34	0.181	0.17	0.313				
Competition vs Level	0.32	0.212	0.39	**0.016**				

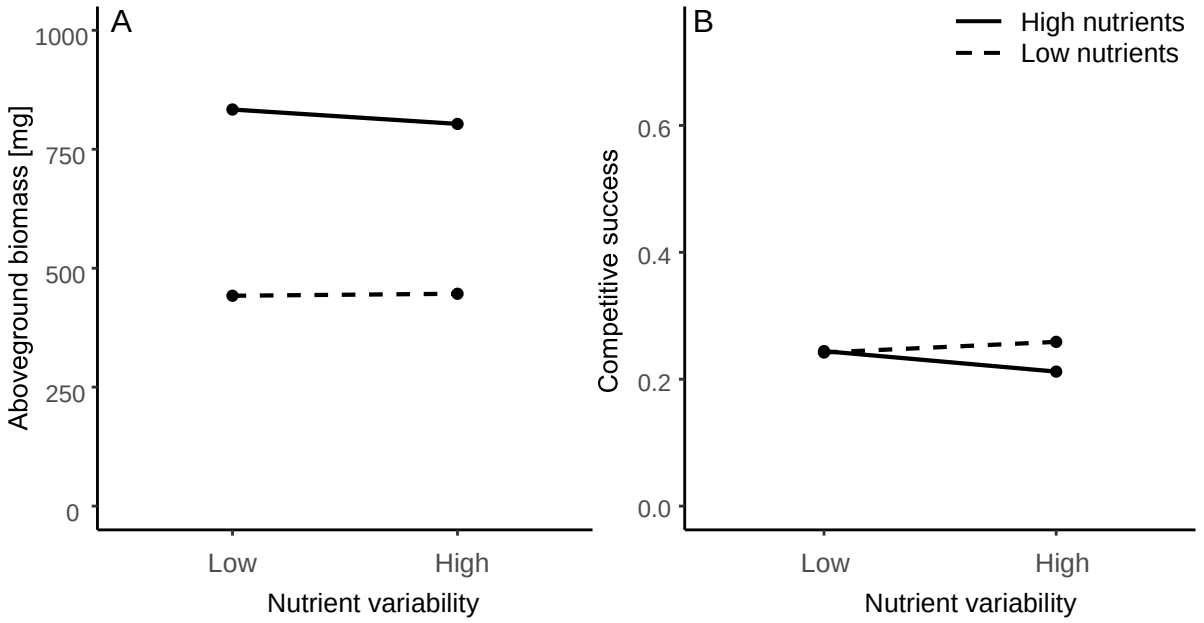

Figure S1. Response of (A) aboveground biomass and (B) competitive success to the interaction of nutrient level and nutrient variability.

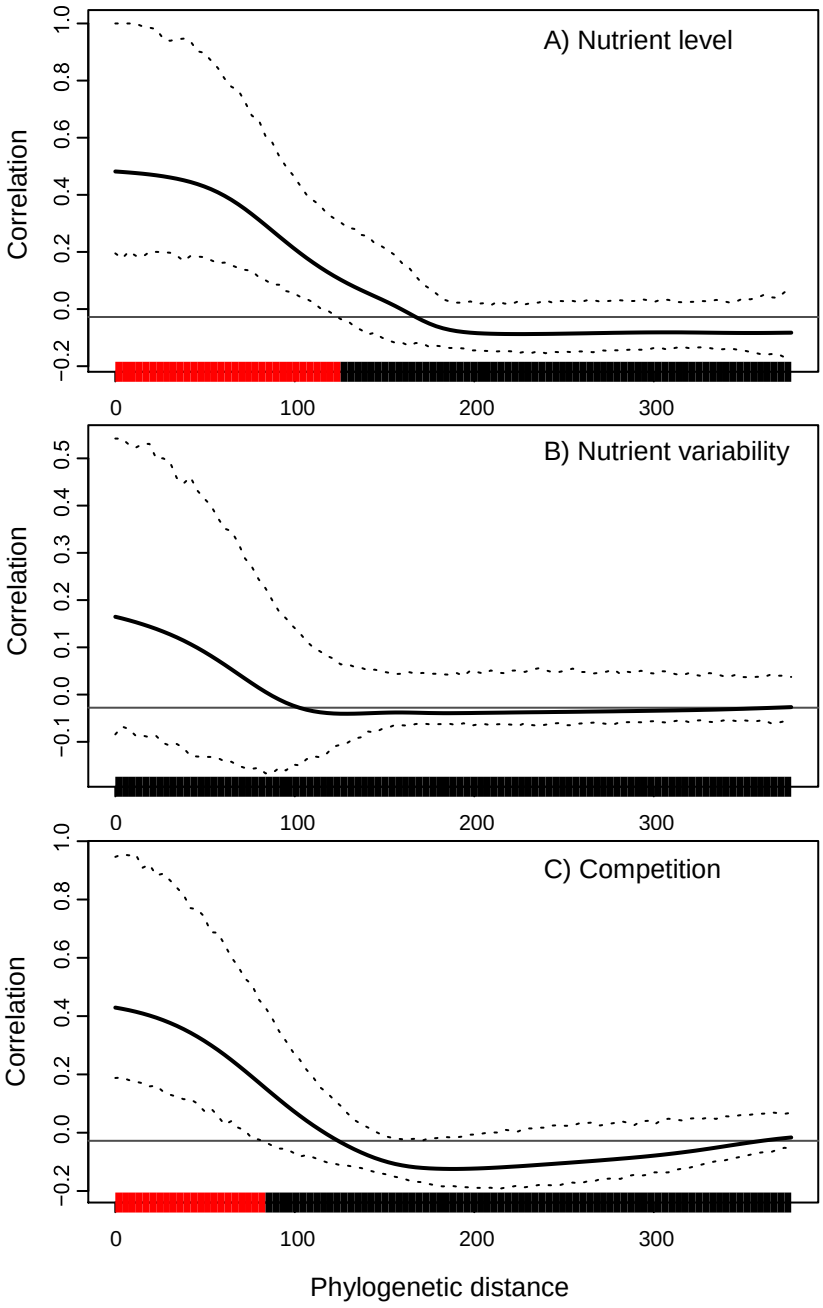

Figure S2. Phylogenetic correlograms for response of aboveground biomass in different treatments. The solid black line represents the Moran's *I* index of autocorrelation at different phylogenetic distance, the dashed lines represent its confidence interval. The horizontal line indicates null hypothesis of no phylogenetic autocorrelation. Significant autocorrelation is indicated by the colored bar, with red for significant positive autocorrelation.

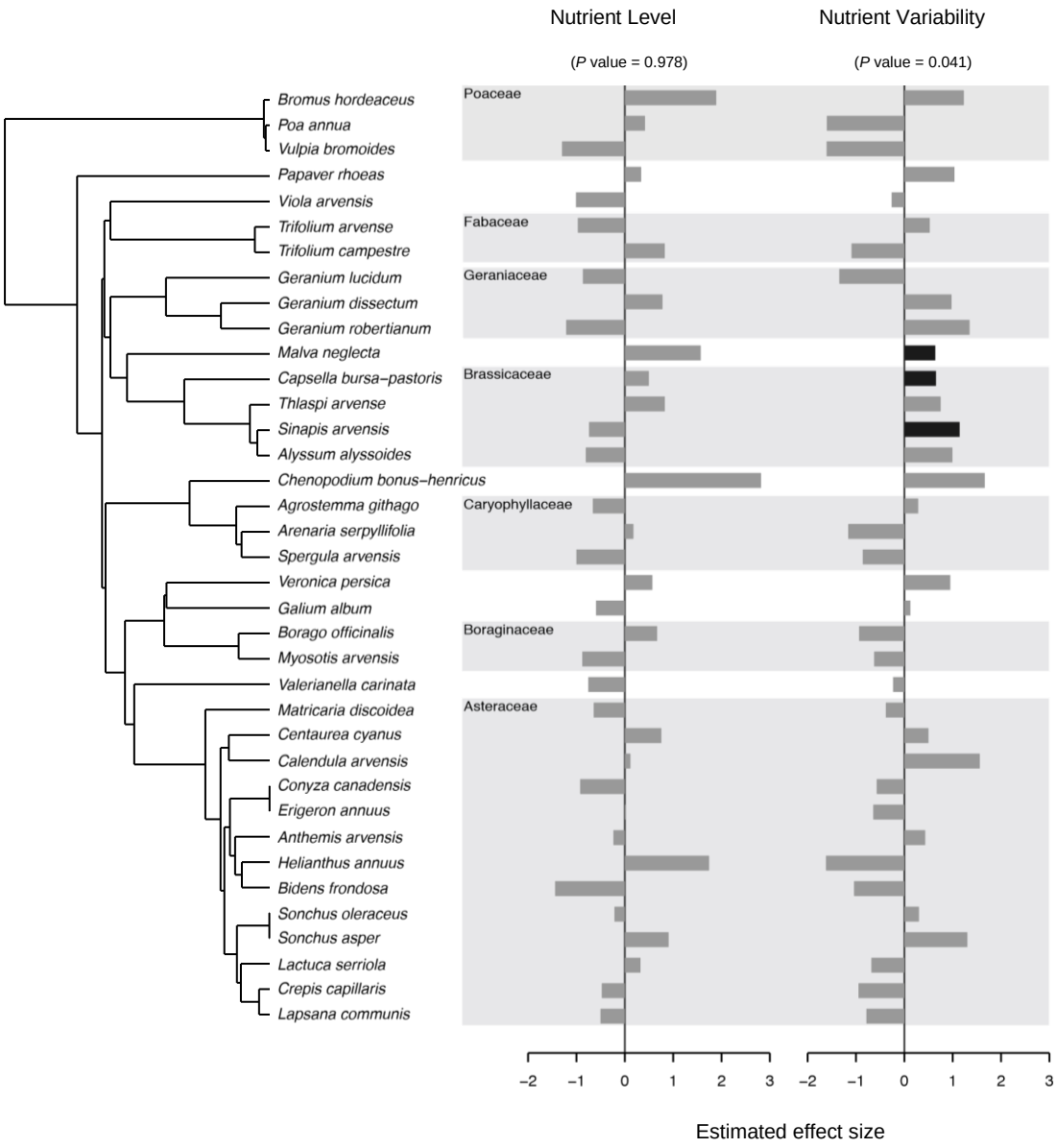

Figure S3. Response of root:shoot ratio to different nutrient treatments mapped along the phylogeny of 37 species. Phylogenetic signal was computed with Abouheif's C_{mean}, *P*-values from respective tests for each treatment were shown on the top. Responses to treatments are shown in bar plots, species were mapped along their phylogeny tree on the left. Dark bars indicate species with significant local Moran's *I* values, showing 'hotspots' of local positive autocorrelation.

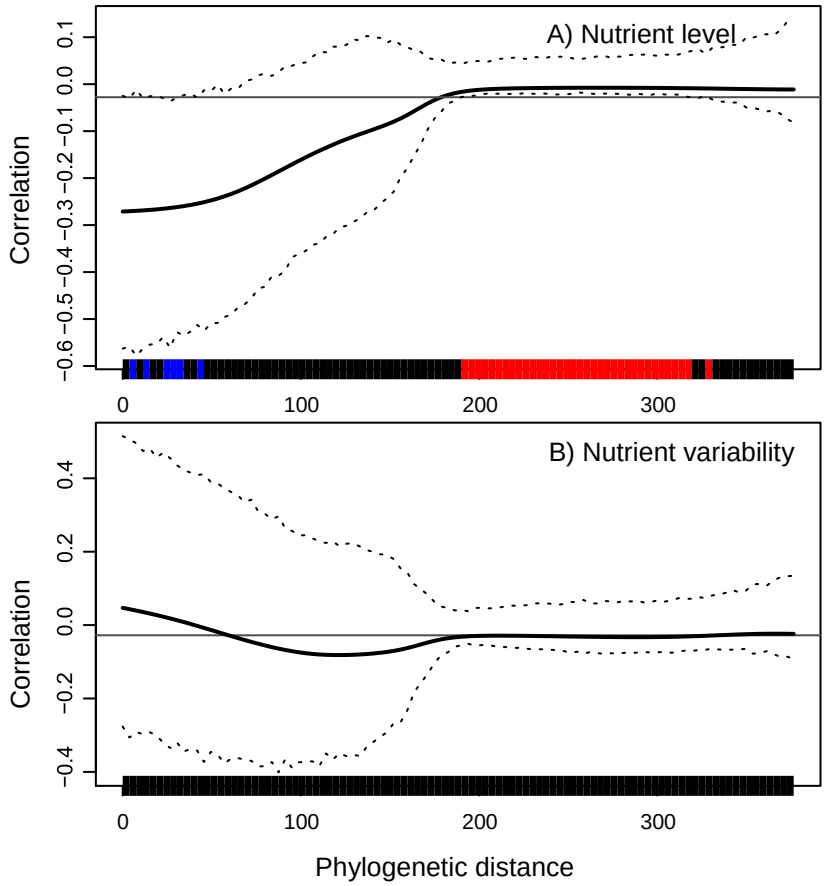

Figure S4. Phylogenetic correlograms for response of root:shoot ratio in different treatments. The solid black line represents the Moran's *I* index of autocorrelation at different phylogenetic distance, the dashed lines represent its confidence interval. The horizontal line indicates null hypothesis of no phylogenetic autocorrelation. Significant autocorrelation is indicated by the colored bar, with red for significant positive autocorrelation, and blue for significant negative autocorrelation.

Chapter IV

Phenotypic plasticity in response to temperature fluctuations is genetically variable, and relates to climatic variability of origin, in *Arabidopsis thaliana*

J.F. Scheepens, **Ying Deng**, Oliver Bossdorf

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Abstract

Under current climate change, increasing mean temperatures are not only causing hotter summers, but temperature variability is increasing as well. Phenotypic plasticity can help plants to overcome negative effects of temperature variability and allow them to rapidly adjust traits to adverse conditions. Moreover, genetic variation in such plasticity could provide potential for adaptive evolution in response to changing climate variability. Here, we conducted an experiment with 11 *Arabidopsis thaliana* genotypes to investigate intraspecific variation in plant responses to two aspects of variable temperature stress: timing and frequency. We found that the timing but not frequency of temperature stress affected the phenology, growth, reproduction and allocation strategy of plants, and that genotypes differed substantially in their responses. Moreover, trait plasticity was positively related to precipitation variability of origin, suggesting an adaptive role of plasticity. Our results indicate that the developmental stage of a plant during heat stress is a key determinant of its response, and that plasticity to temperature variability is an evolving and possibly adaptive trait in natural populations of *A. thaliana*. More generally, our study demonstrates the usefulness of studying plant responses to climatic variability *per se*, given that climatic variability is predicted to increase in the future.

Keywords

adaptation, climatic variability, genotype, heat stress, intraspecific variation, phenotypic plasticity

Introduction

Global climate change is significantly affecting plants and animals across the globe (Parmesan and Yohe 2003; Root et al. 2003; Menzel et al. 2006; Reyer et al. 2013). Under current climate change, increasing mean temperatures are not only causing hotter summers, but temperature variability is increasing as well (Schär et al. 2004; Fischer and Schär 2009). This increase in variability can take place at different temporal scales, e.g. diurnally, intra-seasonally or inter-annually. As a consequence, temperature extremes are currently occurring more regularly and are predicted to increase even more in frequency in the future (Fischer and Schär 2009; Barriopedro et al. 2011).

While plant and community responses to changing mean temperature and precipitation have already been well investigated (Walther et al. 2002; Wu et al. 2011), much less work has been done so far on plant responses to changes in climatic variability (Jentsch et al. 2007; Reyer et al. 2013). Some previous studies indicate that increasing climatic variability *per se* may have strong repercussions for plant and community performance (Knapp et al. 2002; Chesson et al. 2004; Sher et al. 2004) and that climatic variability may sometimes affect population dynamics and community functioning even more strongly than climatic means (Fay et al. 2000; Sher et al. 2004). Moreover, as plant populations are often adapted to their climates of origin (Manel et al. 2010; Fournier-Level et al. 2011; Hancock et al. 2011; Ågren and Schemske 2012; Toräng et al. 2014), and this may include not only adaptation to the means of temperature and precipitation (Manel et al. 2010) but also to their temporal variability (Pratt and Mooney 2013; Manzano-Piedras et al. 2014), climate change may disrupt such adaptations.

If temperature fluctuations and high temperature stress have negative effects on plant growth (Kotak et al. 2007), then the current and predicted increase in the frequency of temperature extremes will impact plant fitness and survival, with potential repercussions on population persistence (Jump and Peñuelas 2005; but see Cahill et al. 2012). However, populations may differ genetically in their tolerance to temperature fluctuations, and such variation may reflect past selection by the climatic variability of the site (Gianoli and González-Teuber 2005; Pratt and Mooney 2013; Lázaro-Nogal et al. 2015). For instance, a study on a semi-arid Chilean shrub, *Senna candolleana*, showed that populations from climatically more variable sites showed greater adaptive plasticity to water availability and may therefore be able to cope better with future increasing climatic variability despite being exposed to higher levels of stress (Lázaro-Nogal et al. 2015). Such intraspecific variation in responses to climatic variability may prove crucial for future adaptation to changing climatic variability, and it suggests that populations in climatically variable environments may suffer less from increasing variability than populations from more stable climatic conditions. A formal proof of adaptive plasticity in response to climatic variability would require to demonstrate positive relationships between the degree of plasticity across different climates and the mean fitness across these environments (Sultan 2000; Relyea 2002; van Kleunen and Fischer 2005).

Climatic variability is a broad term, and a change in variability may have different aspects. For instance, for discrete environmental events, variability may change through changes in the events' duration, frequency, timing and/or intensity (Shea et al. 2004). Each of these aspects may have different effects on the organisms, and experiments allow us to control and study them separately. Whatever the exact experimental design is, an important notion is that experiments investigating the effects of changes in climatic variability should avoid confounding changes in the variability of a climate variable with changes in its mean by keeping the overall mean of an experimentally altered climate variable, e.g. the average

temperature or precipitation sum, constant across the experiment (Parepa et al. 2013), or by combining changes in means with changes in variability in a multi-factorial experimental design. So far, such experiments are still rare.

Here, we conducted an experiment in which we investigated intraspecific variation in plant responses to two aspects of variable temperature stress: timing and frequency. We used *Arabidopsis thaliana* as a model system, because natural genotypes from various geographic locations with contrasting climates are readily available from seed stock centers and these exhibit large genetic variation (1001 Genomes Consortium 2016). In general, genotype-environment interactions and their genetic basis have already been well-studied in *A. thaliana*. For instance flowering time responses across 473 natural genotypes grown under two contrasting temperature and light environments mimicking Spanish and Swedish climates suggest adaptation (Li et al. 2010), and this result has been corroborated in a field study in Italy and Sweden (Ågren and Schemske 2012). Vile and co-workers (2012) found variable responses to temperature and drought treatments in various traits among ten natural genotypes. The production of heat shock proteins (HSPs) in response to heat stress was found to be variable among genotypes and related to heat-stress resistance as well as to heat-stress levels experienced under natural conditions (Tonsor et al. 2008). Thus, genotype by environment interactions are abound in *A. thaliana*, but virtually all studies investigated responses to changes in environmental means whereas studies on genotype-specific responses to changes in environmental variability are so far lacking.

We used eleven *A. thaliana* genotypes from the species' natural range, and exposed the plants to six different scenarios of temporally variable temperature stress while keeping the average temperature constant across all treatments. The overall aim of the study was to investigate how plants responded in terms of performance, phenology and architecture to changes in the timing versus frequency of temperature stress, and whether there was intraspecific variation in these responses that would indicate evolutionary potential for adapting to changing climatic variability. We also tested whether plasticity to temperature variability was adaptive, and whether it was related to the climate of origin of the 11 studied genotypes. Specifically, we asked the following four questions: (1) How does *A. thaliana* respond to changes in the timing versus frequency of temperature stress? (2) Do genotypes differ in their responses to these changes? (3) If yes, is the degree of plasticity to the different temperature stress treatments related to the fitness robustness of *A. thaliana* genotypes across environments? (4) Is the tolerance of *A. thaliana* genotypes to temperature stress related to their climatic origin?

Methods

Experiment

To examine tolerance to temperature variability, and genetic variation therein, of *A. thaliana*, we performed a full-factorial experiment in which 11 *A. thaliana* genotypes were subjected to temperature stress at different times and frequencies. We initially selected 12 genotypes from the Versailles 'core collections' maximizing genetic diversity (McKhann et al. 2004; Table S1). Specifically, we worked with Blh-1, Bur-0, Ct-1, Ita-0, JEA, Oy-0 and Sha from the 'core collection 8', plus Can-0, Ge-0, Mt-0, N13 and St-0 from the 'core collection 16'. All selected lines were of native origin and did not require vernalization to flower. During our experiment,

all plants from the genotype Ita-1 (but none of the others) died of an unidentified fungal disease and were therefore removed from the experiment and subsequent analyses, leaving 11 genotypes.

We placed seeds from all genotypes on moist filter paper in Petri dishes and stratified them for five days at 4 °C in the dark. Thereafter, we sowed the seeds into 5×5×4.5 cm pots filled with a 45:45:10 mixture of potting soil, low-nutrient germination soil (Einheitserde, Sinntal-Altengronau, Germany) and sterilized sand. We initially planted two seeds of the same genotype in each pot, with 59 pots per genotype, and 708 pots in total. Prior to the start of the experimental treatments, we thinned down all plants to one seedling per pot.

For our experiment, we used two walk-in growth chambers that were identical except for their temperature settings. The "normal" chamber was set to 20/15 °C at a 14/10 hours day/night cycle, whereas the "stress" chamber was set to 30/25 °C with the same light conditions. The day temperature of 30 °C experienced in the stress chamber is known to exert stress on *A. thaliana* (Whittle et al. 2009; Vile et al. 2012), and this was confirmed in our experiment where periods spent in the stress chamber often resulted in aborted flowers and fruits. Under day conditions, the light intensity in the growth chambers was ca. 230 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetically active radiation. Air moisture was kept within 40-60%.

One set of control plants, with eight replicates per genotype, was placed in the normal chamber, while another set of control plants, with three replicates per genotype, was placed in the stress chamber for the whole duration of the experiment. The remaining 48 plants per genotype were all subjected to the same amount of 12 days of temperature stress, but with different temporal patterns of the stress periods, which were achieved by moving the plants from the normal chamber to the stress chamber at different times. Besides the two controls, there were six different stress treatments (Fig. 1): a factorial combination of three different timings of stress (early/intermediate/late) and two different frequencies (low/high), with eight replicates per genotype in each treatment. After a one-week establishment phase for all plants in the normal chamber, the early-stress plants were moved to the stress chamber at day 8, and the intermediate- and late-stress plants at days 26 and 44, respectively. For each of these timing treatments, we imposed temperature stress at two different frequencies, either with two periods of six days of stress, and six days of recovery at normal conditions in between, or with four periods of three days of stress, and three days of recovery between each of these (Fig. 1). After the late-stress period, all plants except the control plants in the stress chamber remained in the normal chamber until they were harvested.

Throughout the experiment, we watered all plants regularly, so that water presumably never became a limiting factor. Every morning, we recorded the phenological state of each plant. The plants were classified as flowering when the first flower opened. At the end of the intermediate-stress period (day 43), we took leaf samples from a subset of the early- and intermediate-stress plants for subsequent molecular analyses (not reported here). Each plant was harvested one week after the first fruit ripened. We counted the numbers of fruits >2 mm as well as the numbers of basal and lateral shoots. We separated aboveground vegetative biomass (the rosette) from the reproductive biomass (inflorescences). The vegetative biomass was immediately dried for 72 hours at 60 °C and weighed, whereas the inflorescences were first stored at room temperature for after-ripening and seed harvesting (for follow-up experiments) and then also dried and weighed.

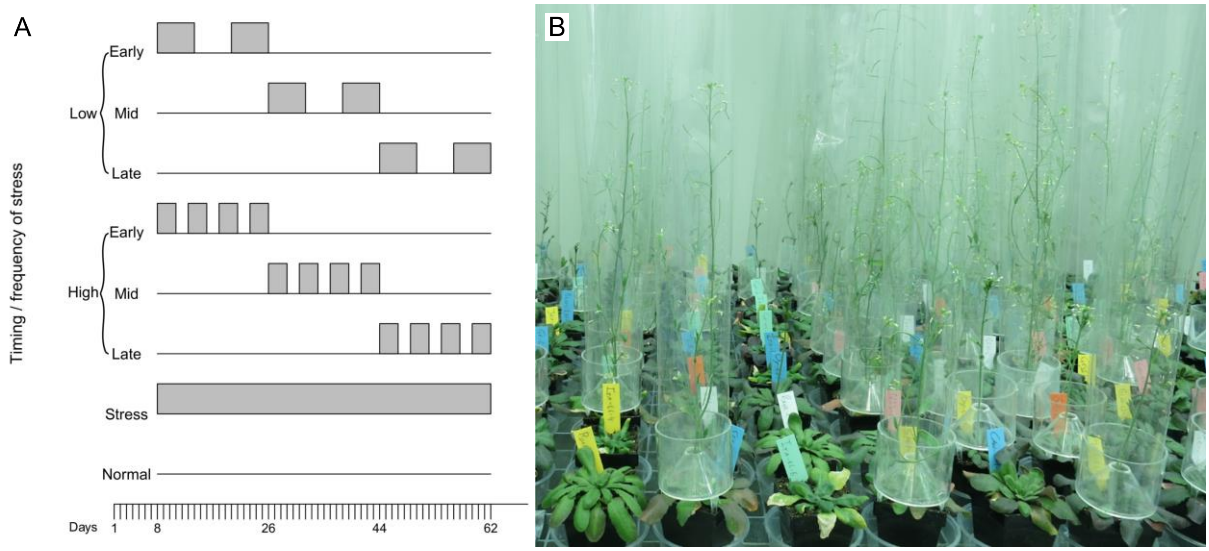

Figure 1. (A) Schematic of the six temperature fluctuation treatments – with three timings (early/mid/late) and two frequencies (low/high) of temperature stress – and two continuous control treatments at normal and stressful temperature. The grey blocks indicate the periods during which the plants experienced temperature stress. (B) Close-up of some of the experimental plants (Photo: JF Scheepens).

Data analysis

We analysed plant responses to temperature stress with regard to the following five response variables: (1) flowering time, (2) plant architecture, i.e. the ratio of lateral to basal shoot number, with lower numbers indicating more 'shrubby' plants, (3) aboveground biomass, (4) reproductive allocation, i.e. the proportion of reproductive to total aboveground biomass, and (5) fecundity, i.e. the number of fruits. To account for the biomass removal through leaf sampling from some early- and intermediate-stress plants, we included leaf sampling as a binary variable in all analyses.

First, we verified our experimental treatments, and whether the stress chamber conditions were indeed stressful for the plants, by analysing only the fecundity of the plants in the continuous normal versus continuous stress conditions. In this linear model, we also tested for genotypic differences in fecundity, and for the interaction between genotype and the continuous temperature treatments.

Next, we analysed the data from the six temperature fluctuation treatments with linear models that included leaf sampling, genotype, timing and frequency of stress as well as all possible two-way and three-way interactions between genotype, stress timing and stress frequency. To improve normality of the model residuals, flowering time was log-transformed and plant architecture was square root-transformed prior to the analyses.

To investigate whether increased trait plasticity is associated with higher robustness in terms of plant fitness, we used linear regressions that related a standardized measure of fitness robustness of each genotype across environments to its trait plasticity across environments. To calculate fitness robustness, we divided the mean fitness across the six treatments by the maximum fitness achieved in one of the six treatments. This index renders the genotypes comparable among each other. The degree of trait plasticity was quantified

using the coefficient of variation based on the mean trait values in the six treatments (Valladares et al. 2006).

Finally, we tested whether the observed degree of trait plasticity of different genotypes was related to their climate of origin. We used temperature and precipitation data from WorldClim (Hijmans et al. 2005) and calculated, for each genotype, the mean and standard deviation of temperature as well as the mean and coefficient of variation (CV) of precipitation for the months of the growing season. For each genotype's location of origin, the growing season was determined based on threshold monthly values of minimum (> 5 °C) and maximum temperature (< 30 °C), precipitation (> 20 mm month^{-1}) and water deficit (> -50 mm month^{-1}), with water deficit calculated as precipitation minus evapotranspiration, and evapotranspiration calculated according to Droogers and Allen (2002). In case all four thresholds were met for a given month, this month was included in the growing season and the calculation of climate variables. The growing season was, however, fixed to a length of four months starting with the earliest suitable month (Table S1).

All analyses were performed in the software R v 3.4.3 (R Core Team 2017).

Results

Plants continuously growing in the stress chamber had a significantly lower average fecundity (159.6 ± 24.0) than the plants continuously growing in the normal chamber (169.0 ± 12.8; ANOVA, $F_{1,85} = 5.48$, $P = 0.022$), confirming that the higher temperatures in our experiment indeed exerted significant stress and decreased plant fitness. However, the overall effect of temperature stress differed among genotypes ($F_{8,85} = 2.22$, $P = 0.034$), with some genotypes showing strong negative responses and others showing only weak or no responses, and one genotype even showing a positive response (Fig. S1).

The analyses of the plants in the six variable stress treatments showed that overall, timing but not frequency of temperature stress affected performance, phenology and architecture of the plants (main effect of stress timing and its interaction with genotype; Table 1). Across all genotypes, the timing of stress significantly affected fecundity as well as reproductive allocation and plant architecture, with the highest average fecundity and the lowest ratio of lateral to basal shoots in early-stressed plants, and lowest reproductive allocation at intermediate stress timing (Fig. 2). However, some individual genotypes deviated from these general trends. We also found significant interactions between stress frequency and timing in fecundity and reproductive allocation: higher frequency had a positive effect on both of these traits if the stress occurred early, but it had no or even the opposite effect if the stress occurred later (Fig. S2). There were strong genotype effects in all of the measured traits, and the effects of stress timing were also generally strongly genotype-dependent (Table 1, Fig. 2). Finally, there was a three-way interaction among stress timing and frequency, and genotype identity for reproductive allocation (Table 1), which therefore modulates the two-way interaction of stress timing and frequency (Fig. S3). Results hardly differed when plants which leaves were sampled for subsequent molecular analyses were removed from the analysis (Table S2).

Trait plasticity was negatively related to fitness robustness for flowering time ($F_{1,9} = 10.68$, $P = 0.010$; Fig. 3A), plant architecture ($F_{1,9} = 5.97$, $P = 0.037$; Fig. 3B), aboveground biomass ($F_{1,9} = 16.71$, $P = 0.003$; Fig. 3C) and reproductive allocation ($F_{1,9} = 10.21$, $P = 0.011$; Fig. 3D). When relating trait plasticity to the climates of genotype origin, we found that for four out of

five traits (i.e. all except aboveground biomass), trait plasticities were positively related to the precipitation variability of origin (Table 2; Fig. 4). Except for one significant positive relationship of plant architecture with mean precipitation of origin (R^2_{adj} = 0.32; $F_{1,9}$ = 5.70, P = 0.041; Table 2), trait plasticity was unrelated to any of the other climate variables.

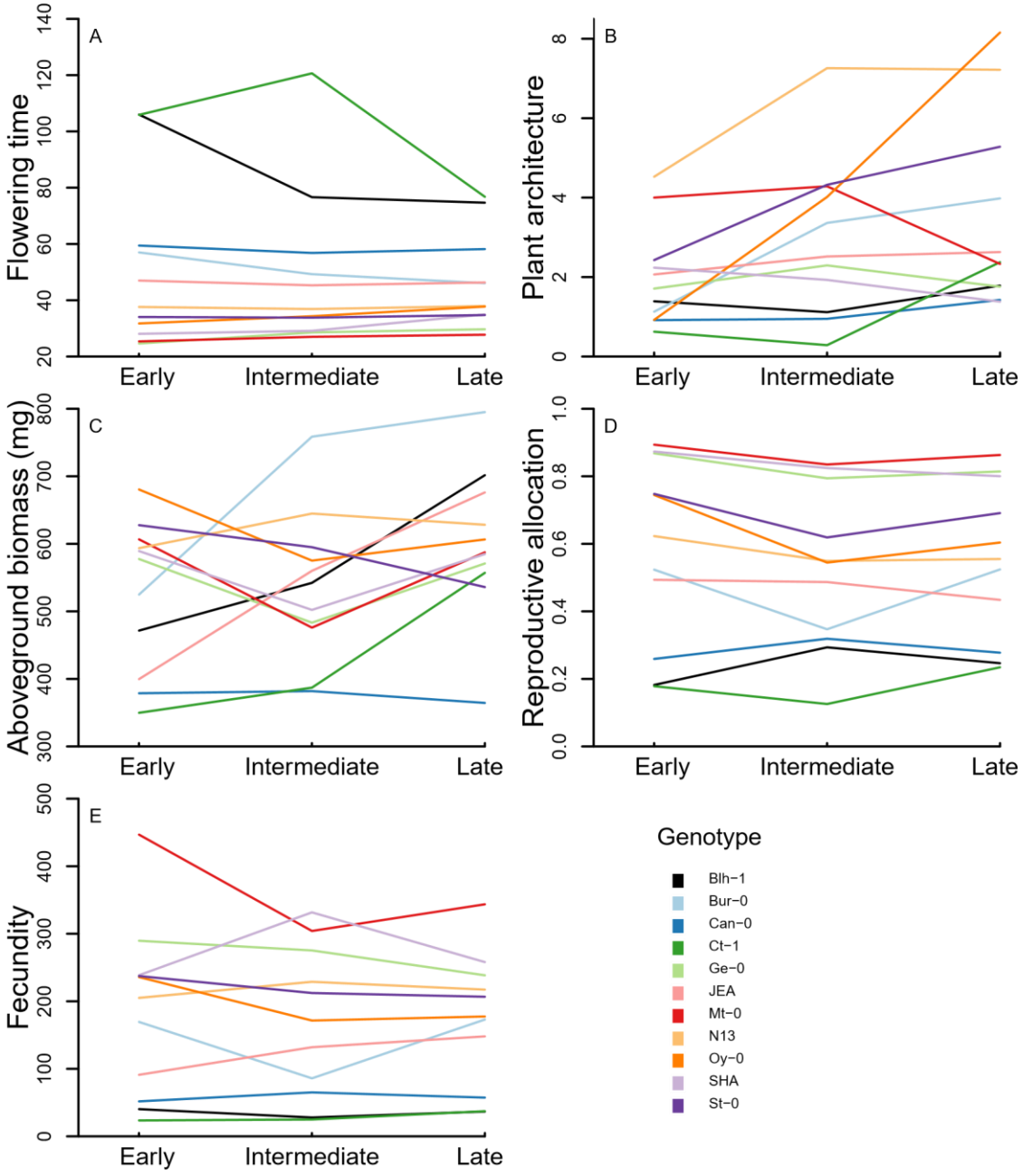

Figure 2. Response of 11 *Arabidopsis thaliana* genotypes to three different timings of temperature stress in five traits: (A) flowering time; (B) plant architecture; (C) aboveground biomass; (D) reproductive allocation; (E) fecundity.

Table 1. Results of linear models testing the phenotypic responses of 11 *Arabidopsis thaliana* genotypes to different timings (early/mid/late) and frequencies (low/high) of temperature stress. Shown are *F*-ratios and *P*-values, the latter highlighted in bold when below 0.05.

		Flowering time		Plant architecture		Aboveground biomass		Reproductive allocation		Fecundity	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Leaf sampling	1	0.39	0.533	9.70	**0.002**	69.05	**< 0.001**	2.27	0.133	1.47	0.226
Stress Timing (T)	2	0.22	0.805	20.31	**< 0.001**	2.67	0.071	14.74	**< 0.001**	3.23	**0.041**
Stress Frequency (F)	1	1.46	0.227	0.01	0.931	1.04	0.308	0.83	0.364	0.68	0.409
T × F	2	1.67	0.189	0.37	0.692	0.85	0.428	6.74	**0.001**	5.66	**0.004**
Genotype (G)	10	356.23	**< 0.001**	45.19	**< 0.001**	23.61	**< 0.001**	297.90	**< 0.001**	131.11	**< 0.001**
G × T	20	7.97	**< 0.001**	6.53	**< 0.001**	6.63	**< 0.001**	4.73	**< 0.001**	4.99	**< 0.001**
G × F	10	1.59	0.107	1.54	0.123	0.65	0.769	0.82	0.609	0.73	0.694
G × T × F	20	0.78	0.743	1.18	0.265	1.34	0.148	2.47	**< 0.001**	0.87	0.621
Residuals	447-454										

Table 2. Results of linear regressions testing for relationships between the climates of origin of 11 *Arabidopsis thaliana* genotypes, and their trait plasticities in response to fluctuating temperature stress. Shown are adjusted R^2-values, *F*-ratios and *P*-values, the latter highlighted in bold when below 0.05.

	Plasticity														
	Flowering time			Plant architecture			Aboveground biomass			Reproductive allocation			Fecundity		
	R^2_{adj}	*F*	*P*	R^2_{adj}	*F*	*P*	R^2_{adj}	*F*	*P*	R^2_{adj}	*F*	*P*	R^2_{adj}	*F*	*P*
Mean temperature	-0.10	0.06	0.806	-0.11	0.05	0.831	0.0	0.96	0.353	0.23	4.03	0.076	-0.08	0.23	0.645
SD of temperature	-0.11	0.02	0.884	0.02	1.16	0.309	-0.11	0.02	0.895	0.02	1.21	0.300	-0.08	0.22	0.647
Mean precipitation	0.14	2.64	0.139	0.32	5.70	**0.041**	-0.11	0.02	0.887	-0.03	0.71	0.423	-0.03	0.70	0.424
CV of precipitation	0.64	18.57	**0.002**	0.59	15.64	**0.003**	0.27	4.65	0.059	0.74	29.82	**<0.001**	0.47	9.69	**0.012**

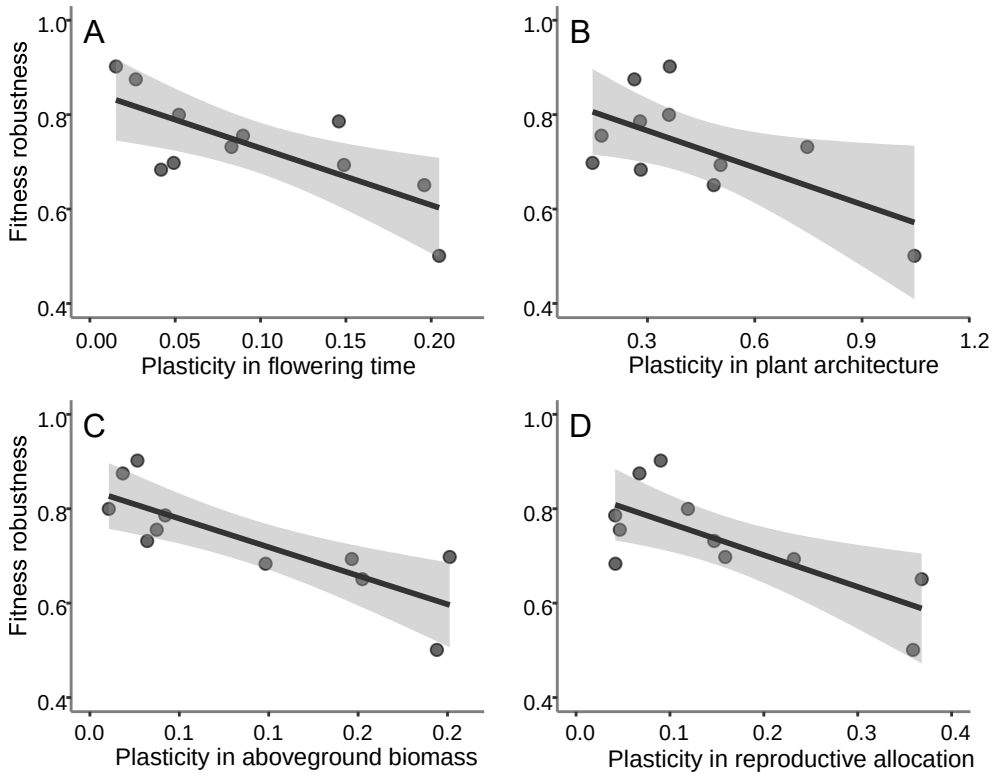

Figure 3. Relationships between fitness robustness across environments and trait plasticity – (A) flowering time; (B) plant architecture; (C) aboveground biomass; (D) reproductive allocation – for 11 genotypes of *Arabidopsis thaliana*.

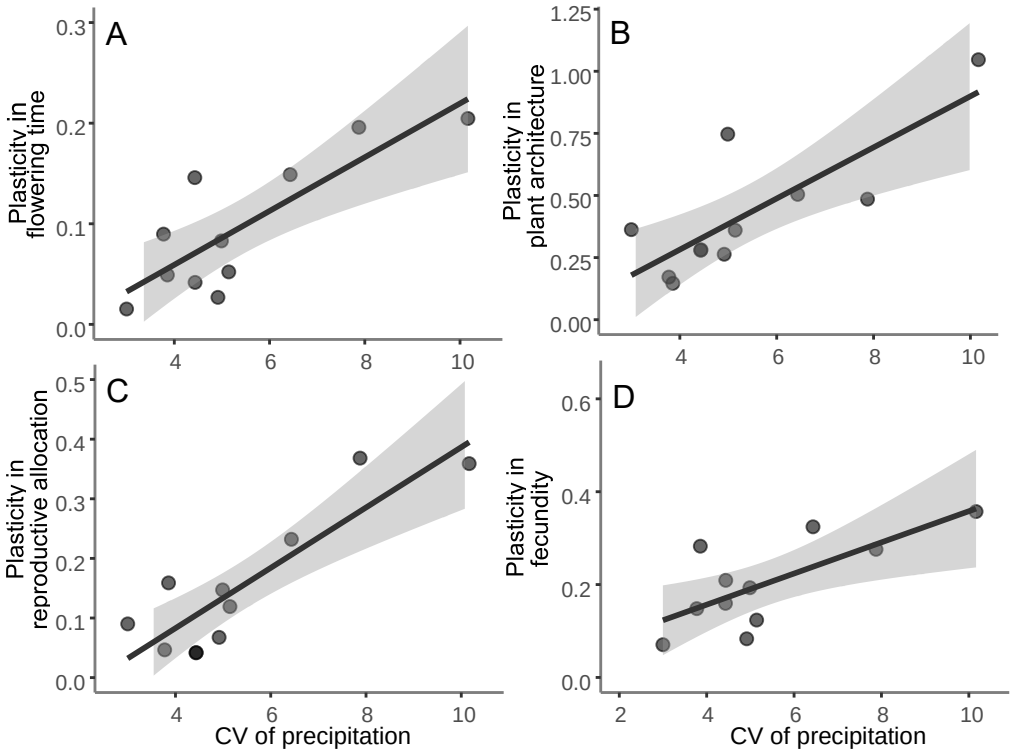

Figure 4. Relationships between trait plasticity – (A) flowering time; (B) plant architecture; (C) reproductive allocation; (D) fecundity – and precipitation variability of origin for 11 genotypes of *Arabidopsis thaliana*.

Discussion

The goal of our study was to better understand how plants respond to changes in the temporal variability of the environment, and the extent and structure of intraspecific variation in this respect. We found that the timing of temperature stress strongly affected the growth and reproduction, resource allocation, phenology and architecture of *A. thaliana*, but the frequency of temperature stress did not. There was large variation in plasticity to stress timing among the 11 tested *A. thaliana* genotypes, and their degree of plasticity in this experiment was negatively related to fitness robustness, but positively related to the precipitation variability of their origins. Below, we discuss each of these results, and their implications, in detail.

Timing, not frequency, of temperature stress matters

Arabidopsis. thaliana responded to different timing but not to frequency of temperature stress. It is likely that the observed effects of stress timing were related to plant development. The developmental stage is important for a plant's response to heat stress (Wollenweber et al. 2003; Hedhly et al. 2009). For instance, Wollenweber and co-workers (2003) found that heat stress did not affect wheat yield when applied during the vegetative stage but caused strong yield declines when applied during flowering. Similarly, we found that plants that were flowering during a stress treatment tended to abort these flowers (personal observation), leading to reduced fitness. Nine out of eleven genotypes started flowering during days 24-60, i.e. largely during the period when the intermediate and late stress treatments were applied to some of the plants, and the remaining two genotypes started flowering after all treatments were done; virtually no flowering took place during early stress. This may explain the overall reduction in fecundity under intermediate and late when compared to early temperature stress. Nevertheless, results for fecundity, aboveground biomass, reproductive investment and plant architecture did not change when we added flowering time as a covariate in the models (Table S3). Perhaps other developmental stages, such as flowering duration, are more important determinants of the outcome of stress timing on plant traits.

The absence of an effect of stress frequency may be explained by an acquired thermotolerance, where after initial exposure to temperature stress, thermotolerance is retained, or decaying only slowly over time (Burke et al. 2000; Charng et al. 2006). This could explain why a different number of subsequent exposures to stress does not lead to a different response. The mechanism underlying acquired thermotolerance could be HSPs. It is well known that plants produce HSPs after exposure to high temperatures (Kotak et al. 2007), and that HSPs play a central role in heat stress resistance through their function as molecular chaperones, i.e. they stabilize other proteins and thereby safeguard their functioning (Sørensen et al. 2003; Kotak et al. 2007).

Genotypic variability

All traits showed substantial genotypic variation in their responses to timing of stress. As explained above, plants often respond differently to environmental stimuli depending on the developmental stage they are in (Hedhly *et al.* 2009). Since the genotypes in this experiment differed in their developmental rates, as evidenced by the variation in flowering time, this likely explains part of the genotypic variation in the response to timing of temperature stress

observed in our experiment. Nevertheless, not all genotypic variation can be explained by the phenological stage during stress treatments. For instance, three genotypes (Bur-0, Can-0 and JEA) which started flowering during days 44-62 (i.e. the period of late stress) did not show decreased fitness when they were subjected to heat stress during flowering; JEA even increased fitness and Bur-0 showed a fitness decrease when it received stress at the intermediate timing, before flowering. Contrasting responses in terms of fitness were also observed in the six genotypes which all flowered primarily during the intermediate stress timing, with two genotypes increasing (N13, Sha), three decreasing (Mt-0, Oy-0, St-0) and one hardly responding (Ge-0) to intermediate as compared to early stress. In line with this genotypic variation, adding flowering time as a covariate in the models of the other four traits did not lead to loss of the genotype by stress timing interaction and therefore could not explain the results (Table S3). Thus, genotypes vary in the sensitivity of their reproductive phase to heat stress, and other developmental stages than flowering can be sensitive to heat stress, too. Such variation could, for instance, be related to genotypic differences in HSP genes and activity (Sørensen et al. 2003). Genotypes from more southern latitudes are likely to be naturally exposed, and therefore adapted, to the applied temperature stress treatment in contrast to genotypes from more northern latitudes (Li et al. 2010; Ågren and Schemske 2012). However, adding latitude as a covariate in the models did not lead to loss of the genotype by stress timing interaction (Table S4), so genotypic clines with latitude therefore do not fully explain these genotypic responses.

Whether mediated through developmental stage or through other mechanisms, our results clearly indicate that there is substantial genotypic variation within *A. thaliana* in the response to timing of heat stress. This variation is heritable and therefore constitutes evidence for evolutionary potential which could in principle lead to adaptation to different environments with contrasting temporal patterns of heat stress. However, we should note that the genotypes used in this study originated from diverse geographic locations, so the observed variation likely overestimates the levels of variation within natural populations (where evolution by natural selection takes place). Nevertheless, natural populations of *A. thaliana* are usually not genetically uniform (Bomblies et al. 2009; Montesinos 2009), offering potential for adaptation. Moreover, seed dispersal may to some degree allow adaptive genotypes to track favourable climates. Overall, given projected climate change, it is likely that the timing of heat stress, rather than its frequency, will exert selection pressures on natural populations and result in rapid evolution of their phenotypic plasticity.

Relationship between fitness robustness and plasticity

The negative relationship between fitness robustness and the width of plasticity across the treatments indicates that more plastic genotypes have less stable fitness across environments. In other words, genotypes with stronger trait plasticity suffered on average greater reduction in fitness across environments compared to their optimum (in this experiment), whereas genotypes with weaker plasticity had more robust fitness across environments. It may be that these plant responses to the variable temperature stress treatments are merely passive (e.g. reduced growth under abiotic stress) and go together with a fitness loss. Alternatively, plasticity could be beneficial but costly (Ghalambor et al. 2007). Indeed, HSPs are resource demanding and are toxic at high concentrations (Hoffmann 1995; Feder and Hofmann 1999). Ghalambor and co-workers (2007) described that strong fitness loss may result when an

otherwise adaptive response becomes maladaptive when it falls outside the usually experienced range of environments. However, the two temperature treatments applied in this experiment do not constitute extreme environments for most if not all of the genotypes, rendering this explanation unlikely.

Alternatively, the results may reflect an advantage of phenotypic robustness in the face of the experimentally applied environmental variability, whereas phenotypic plastic responses, whether passive or active, cause fitness losses, at least in this experiment. This may relate to the temporal grain of environmental changes being too fast for plastic responses to be adaptive (Alpert and Simms 2002). In other words, the short periods under temperature stress in this experiment may penalize more plastic genotypes since their responses may be too slow to track the temporal environmental changes the plants were subjected to. Slow or non-responding genotypes may then achieve a higher fitness across the environments and thus be better adapted to such rapid temporal fluctuations in the environment. The question remains, then, whether three or six days of consecutive temperature stress as applied in this study is at odds with heat stress as experienced under natural conditions.

Stronger fitness homeostasis in phenotypically more robust genotypes could also indicate that these genotypes are able to achieve strong plastic responses at the physiological level (Thompson 1991). This seems to be at odds with the positive relationships between plasticity and precipitation variability of origin, which suggest adaptive plasticity of the observed traits.

Relationship between plasticity and climate of origin

We observed that genotypes originating from environments with stronger precipitation variability showed stronger plasticity in most analysed traits. Such relationships fit the classical expectation that more heterogeneous environments should select for more plastic genotypes (Alpert and Simms 2002). It makes theoretical sense that plants in more temporally variable environments are able to adjust reproductive allocation, flowering time and plant architecture more flexibly (Alpert and Simms 2002). For instance, a drought spell may trigger an escape strategy in annuals (Franks 2011), advancing flowering to secure reproduction despite a strong fitness reduction compared to an otherwise more benign environment. The experience of drought may also translate into an altered reproductive allocation and an altered plant architecture (Williams and Black 1994). A key role for variability in water availability was likewise found in studies on other plant species (Gianoli and González-Teuber 2005; Pratt and Mooney 2013; Lázaro-Nogal et al. 2015). Since in the current experiment, plants were well-watered, their responses should therefore not be directly related to drought but rather to temperature stress. Nevertheless, mechanisms and genetic pathways responding to drought and heat stress show considerable overlap in *A. thaliana* (Rizhsky et al. 2004). Heat stress in our experiment could therefore have partially triggered responses that in nature co-occur during drought stress, which may have driven evolution of plasticity. This could explain why the trait plasticities correlated with precipitation variability and not temperature variability of origin: precipitation variability may have been the selective agent for plastic responses while at the same time such responses can be triggered by temperature variability, even though temperature variability itself did not cause evolution of plasticity. An alternative explanation could be that temperature variability of origin, as derived from monthly mean values, does not capture temperature fluctuations relevant for adaptation to temperature

variability. However, correlations between trait variability and mean diurnal temperature were never significant ($P > 0.238$; results not shown). Finally, it should be noted that our limited sample size of 11 genotypes may have constrained the discovery of further plasticity-environment relationships.

Conclusions

Our study shows that phenotypic plasticity in fitness, growth, resource allocation, phenology and architecture in response to temperature variability - in particular to the timing of temperature stress - is variable among *A. thaliana* genotypes and therefore holds evolutionary potential. The observed cross-genotype relationships between responses to variability and climatic variability of origin suggest that evolution has shaped this type of phenotypic plasticity in the past, and that the observed responses possibly reflect adaptive natural variation. Moreover, variability in plasticity might allow natural populations to continue to evolve plasticity under increasingly variable climates in the future. More generally, our study demonstrates the usefulness of studying plant responses not only to changes in mean climate but also to climatic variability *per se*, which is an important finding, given that climatic variability is predicted to increase in the future.

Acknowledgements

We thank Pauline Eichenseer, Christiane Karasch-Wittmann and Ingrid Astfalk for practical assistance during the experiment and Xavier Picó and two anonymous reviewers for their comments on previous versions of our manuscript. This research was financially supported through a research fellowship of the Alexander von Humboldt Foundation to JFS.

Supplementary information

Table S1. *Arabidopsis thaliana* genotypes used in our experiment, with their IDs in the 1001 Genomes project (ID-1; 1001genomes.org) and the NASC (ID-2; www.arabidopsis.info) and Versailles (ID-3; publiclines.versailles.inra.fr) stock centers. The growing season delimits the months of the year included in the calculation of climate means and variabilities.

Name	ID-1	ID-2	ID-3	Country	Latitude	Longitude	Growing season
Blh-1	-	N1030	180AV	Czech Republic	48.30	19.85	5-8
Bur-0	7058	N1028	172AV	Ireland	54.1	-6.2	5-8
Can-0	7063	N1064	163AV	Spain	29.21	-13.48	11-2
Ct-1	7067	N1094	162AV	Italy	37.51	15.09	12-3
Ge-0	8297	N1186	101AV	Switzerland	46.21	6.14	6-9
Ita-0	-	N1244	157AV	Morocco	34.09	-4.20	11-2
JEA	-	-	25AV	France	43.68	7.33	3-6
Mt-0	-	N1380	94AV	Libya	32.34	22.46	11-2
N13	-	N22491	266AV	Russia	61.36	34.15	6-9
Oy-0	7288	N1436	224AV	Norway	60.39	6.19	5-8
Sha	-	N929	236AV	Tajikistan	38.59	68.79	2-5
St-0	8387	N1534	62AV	Sweden	59.34	18.06	5-8

Table S2. Results of linear models testing the phenotypic responses of 11 *Arabidopsis thaliana* genotypes to different timings (early/mid/late) and frequencies (low/high) of temperature stress on plants which were not sampled for leaves for use in follow-up experiments (see main text). Shown are *F-ratio*s and *P-value*s, the latter highlighted in bold when below 0.05. Novel significant results compared to the original model are underlined.

		Flowering time		Plant architecture		Aboveground biomass		Reproductive allocation		Fecundity	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Stress Timing (T)	2	0.15	0.859	14.36	**< 0.001**	3.06	**0.048**	7.12	**< 0.001**	3.69	**0.026**
Stress Frequency (F)	1	2.05	0.154	0.08	0.776	1.80	0.181	2.31	0.129	0.87	0.352
T × F	2	1.35	0.260	0.08	0.923	0.53	0.590	6.30	**0.002**	6.41	**0.002**
Genotype (G)	10	225.40	**< 0.001**	28.56	**< 0.001**	17.51	**< 0.001**	230.33	**< 0.001**	85.36	**< 0.001**
G × T	20	7.75	**< 0.001**	4.34	**< 0.001**	5.89	**< 0.001**	4.46	**< 0.001**	3.16	**< 0.001**
G × F	10	2.18	**0.019**	1.79	0.062	0.51	0.884	1.26	0.254	0.64	0.779
G × T × F	20	1.22	0.240	1.32	0.165	1.36	0.142	2.06	**0.006**	1.06	0.392
Residuals	279-284										

Table S3. Results of linear models testing the phenotypic responses of 11 *Arabidopsis thaliana* genotypes to different timings (early/mid/late) and frequencies (low/high) of temperature stress including flowering time as a covariate. Shown are *F-ratio*s and *P-value*s, the latter highlighted in bold when below 0.05.

		Plant architecture		Aboveground biomass		Reproductive allocation		Fecundity	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Leaf sampling	1	11.15	**0.001**	68.92	**< 0.001**	1.09	0.296	0.94	0.333
Flowering time	1	305.37	**< 0.001**	77.83	**< 0.001**	2350.00	**<0.001**	889.29	**<0.001**
Stress Timing (T)	2	15.68	**< 0.001**	1.52	0.221	33.39	**< 0.001**	6.19	**0.002**
Stress Frequency (F)	1	0.03	0.854	1.80	0.180	1.76	0.186	2.02	0.156
T × F	2	0.52	0.596	0.67	0.513	6.24	**0.002**	5.77	**0.003**
Genotype (G)	10	22.29	**< 0.001**	20.12	**< 0.001**	89.15	**< 0.001**	48.22	**< 0.001**
G × T	20	6.64	**< 0.001**	5.59	**< 0.001**	3.83	**< 0.001**	5.14	**< 0.001**
G × F	10	1.45	0.155	0.53	0.866	0.31	0.977	0.65	0.767
G × T × F	20	1.18	0.263	1.48	0.084	2.10	**0.004**	1.02	0.436
Residuals	443-446								

Table S4. Results of linear models testing the phenotypic responses of 11 *Arabidopsis thaliana* genotypes to different timings (early/mid/late) and frequencies (low/high) of temperature stress including latitude as a random effect. Shown are *F-ratio*s and *P-value*s, the latter highlighted in bold when below 0.05.

		Number of fruits		Aboveground biomass		Reproductive allocation		Flowering time		Plant architecture	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Leaf sampling	1	1.47	0.226	69.05	**< 0.001**	2.27	0.133	0.39	0.533	9.69	**0.002**
Latitude	1	10.81	**0.001**	105.82	**< 0.001**	17.39	**< 0.001**	4.89	**0.028**	124.25	**< 0.001**
Stress Timing (T)	2	3.18	**0.043**	2.44	0.088	14.79	**< 0.001**	0.23	0.792	19.87	**< 0.001**
Stress Frequency (F)	1	0.68	0.411	1.02	0.313	0.84	0.361	1.43	0.232	0.01	0.918
T × F	2	5.66	**0.004**	0.85	0.428	6.74	**0.001**	1.67	0.189	0.37	0.692
Genotype (G)	10	144.49	**< 0.001**	14.53	**< 0.001**	329.06	**< 0.001**	395.27	**< 0.001**	36.50	**< 0.001**
G × T	20	4.99	**< 0.001**	6.63	**< 0.001**	4.73	**< 0.001**	7.97	**< 0.001**	6.53	**< 0.001**
G × F	10	0.73	0.694	0.65	0.769	0.82	0.609	1.67	0.107	1.54	0.123
G × T × F	20	0.87	0.621	1.34	0.148	2.47	**< 0.001**	0.78	0.743	1.18	0.265
Residuals	447-454										

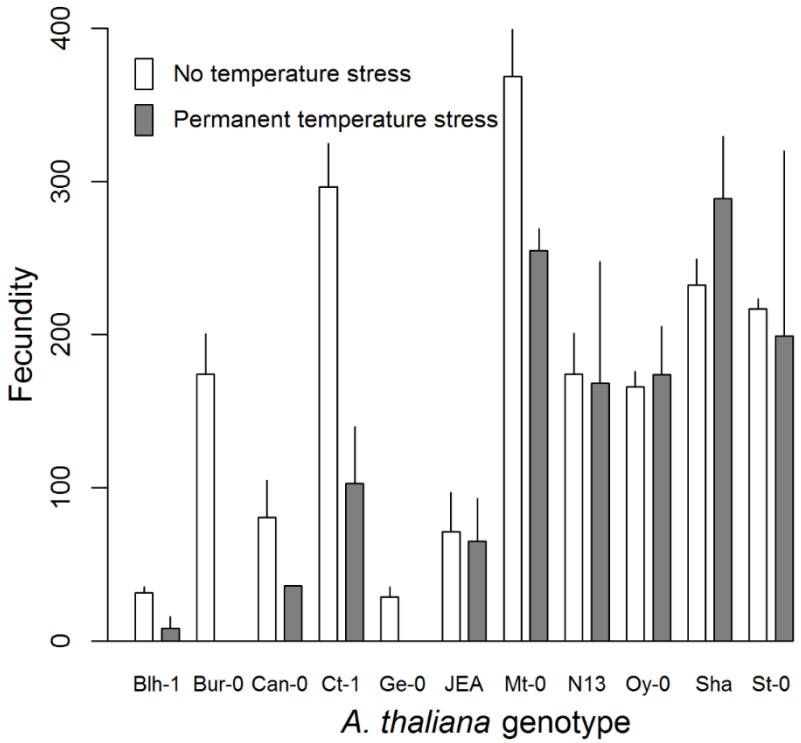

Figure S1. Fecundity of 11 *Arabidopsis thaliana* genotypes under continuous normal conditions (n = 8) and continuous stress conditions (n = 3). Error bars indicate 1 SE.

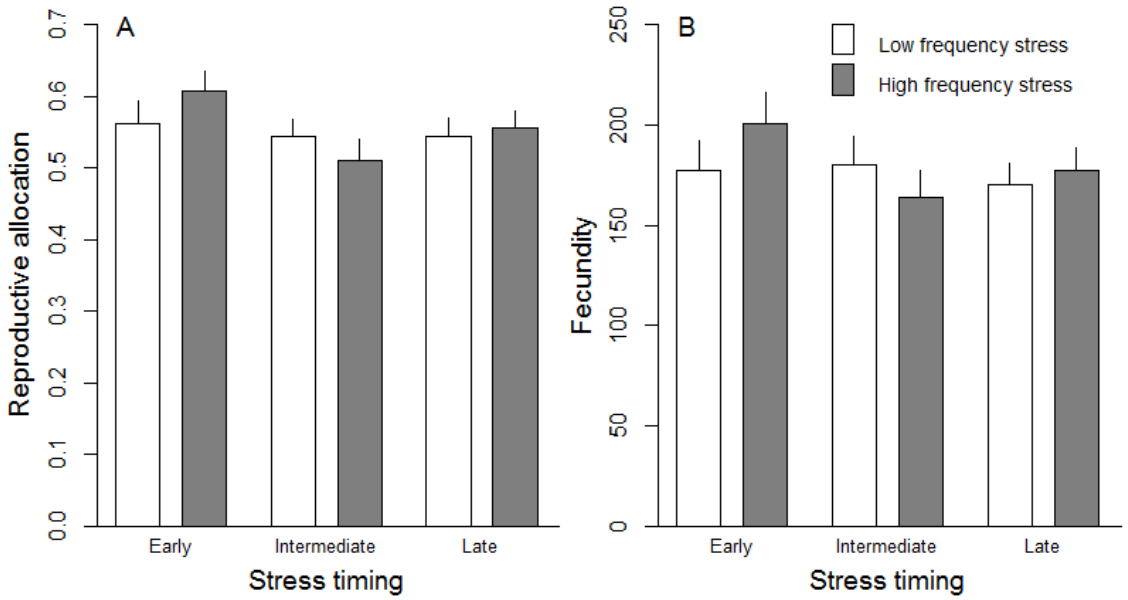

Figure S2. Mean responses of (A) reproductive allocation and (B) fecundity to stress timing and frequency across 11 *Arabidopsis thaliana* genotypes.

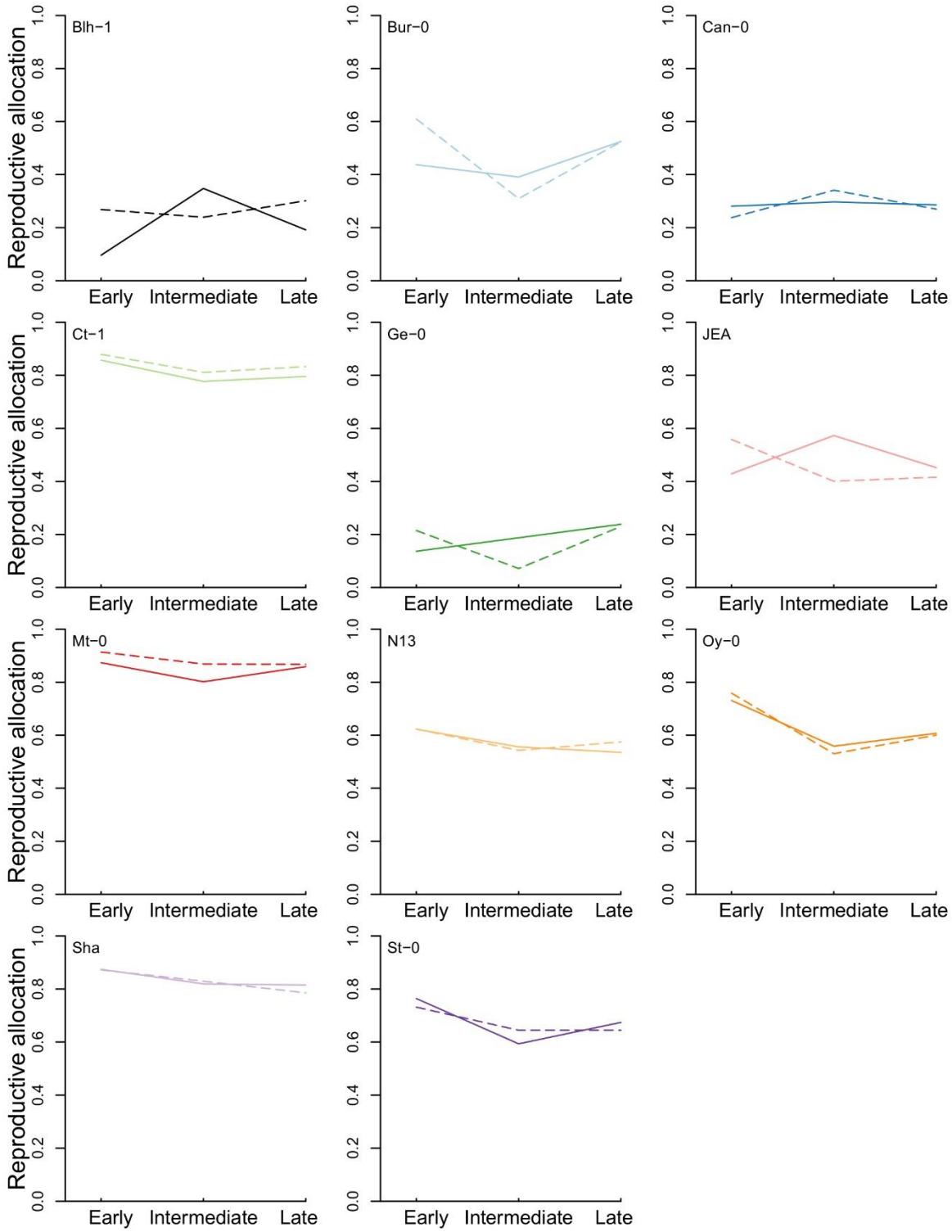

Figure S3. Responses of 11 *Arabidopsis thaliana* genotypes to three different timings and two different frequencies of temperature stress in reproductive allocation. Solid lines indicate responses under low stress frequency and dashed lines under high stress frequency. Genotype names are indicated in the top left corner of the panels.

Chapter V

Transgenerational effects of temperature fluctuations in *Arabidopsis thaliana*

Ying Deng, Oliver Bossdorf, J.F. Scheepens

Abstract

Plant stress responses can extend into the following generations, a phenomenon called transgenerational effects. Heat stress, in particular, is known to affect plant offspring, but we do not know to what extent these effects depend on the temporal patterns of the stress, and whether transgenerational responses are adaptive and genetically variable within species. To address these questions, we carried out a two-generation experiment with nine *Arabidopsis thaliana* genotypes. We subjected the plants to heat stress regimes that varied in timing and frequency, but not in mean temperature, and we then grew the offspring of these plants under controlled conditions as well as under renewed heat stress. The stress treatments significantly carried over to the offspring generation, with timing having stronger effects on plant phenotypes than stress frequency. However there was no evidence that transgenerational effects were adaptive. The magnitudes of transgenerational effects differed substantially among genotypes, and for some traits the strength of plant responses was significantly associated with the climatic variability at the sites of origin. In summary, timing of heat stress not only directly affects plants, but it can also cause transgenerational effects on offspring phenotypes. Genetic variation in transgenerational effects, as well as correlations between transgenerational effects and climatic variability, indicate that transgenerational effects can evolve, and have probably already done so in the past.

Keywords

environmental variability, genetic variation, heat stress, natural variation, phenotypic plasticity

Introduction

Plants encounter various environmental challenges in nature, such as episodes of stressful temperatures or low water availability. Many previous studies have investigated how plants respond to contrasting environmental conditions in terms of their fitness and functional traits (e.g. Sultan et al. 1998; Callahan and Pigliucci 2002; Ibañez et al. 2017; Marais et al. 2017). Although plants generally show reduced fitness under stressful environments, different genotypes often vary in their fitness responses and thus their ability to maintain fitness under adverse environmental conditions (Sultan 1987; Sultan 2000; Ghalambor et al. 2007). Variation in fitness responses is often related to underlying variation in the plasticity of functional traits. For instance, decreased fitness under warmer temperatures may be caused by advanced flowering in the annual *Arabidopsis thaliana* (Ibañez et al. 2017). More generally, there is usually intraspecific variation in plant responses to environmental treatments (i.e. genotype-by-environment interactions, G × E; Sultan 2000; Pigliucci 2001), and if such variation exists within populations, then natural selection can act on it, and the trait plasticity can evolve and adapt to local environmental conditions (Sultan 2000; Groot et al. 2017). If past environments have influenced the evolution of plasticity, we should be able to detect genotype-environment correlations to identify agents of selection shaping plasticity (Groot et al. 2017; Marais et al. 2017).

Organisms may not only respond plastically to their current environments, but their phenotypes may also be influenced by the environmental conditions that their ancestors were exposed to (Uller 2008; Latzel et al. 2014; Groot et al. 2017). This is also called transgenerational plasticity or transgenerational effects. In plants, such transgenerational effects could be physiological and controlled by the mother plant (Herman and Sultan 2011), for instance through endosperm or seed coat modifications, or they could be epigenetic (Whittle et al. 2009; Rasmann et al. 2012; Suter and Widmer 2013) and therefore potentially transferable across even more than one generation (Suter and Widmer 2013; Groot et al. 2017). Through transgenerational effects, plants could prepare (or 'prime') their phenotypes for particular environmental conditions, particularly when offspring are likely to experience similar conditions as their parents, thereby increasing local adaptation (i.e. adaptive transgenerational plasticity; Roach and Wulff 1987; Mousseau and Fox 1998a, 1998b; Agrawal 2001; Galloway 2005; Galloway and Etterson 2007; Uller 2008; Mousseau et al. 2009; Latzel et al. 2014). However, as with regular (within-generation) phenotypic plasticity, transgenerational effects can only evolve as an adaptation when there is genotypic variation in transgenerational effects and when offspring environmental conditions correlate with parental environmental conditions (Uller 2008).

An increasing number of empirical studies with plants investigated how transgenerational effects may confer adaptation particularly under temperature stress (Sultan et al. 2009; Herman and Sultan 2011; Latzel et al. 2014; Groot et al. 2017). For instance, in a single genotype of the model plant *Arabidopsis thaliana*, transgenerational effects of heat stress were observed even in the F3 generations where F3 offspring with the same heat stress in the P1 and F1 generations had a fitness advantage (Whittle et al. 2009). Recently, Groot and co-workers (2017) showed strong genotypic variation in parental and grandparental effects of heat stress in 14 *A. thaliana* genotypes.

So far most studies investigating plant responses to altered and/or stressful environmental conditions - including those studies investigating transgenerational effects - were performed under controlled conditions but usually with stable treatments that did not

consider the temporal variability of environmental stress, which however plays an important role in natural ecosystems (Knapp et al. 2002; Schwinning et al. 2004; Shea et al. 2004). In terms of heat stress, global warming is expected to continue (Giorgi et al. 2004; Barros and Field 2014), but climate anomalies will increase too (e.g. European heat waves in 2003 and 2010), resulting in increasing temporal variability of temperature and, presumably, heat stress (Schär et al. 2004; Fischer and Schär 2009; Barriopedro et al. 2011). For climatic extreme events, the variability aspect itself is often thought to be more important than the involved changes in means (Katz and Brown 1992), and some ecosystems have even been found to be more sensitive to changes in environmental variability than to changes in environmental means (Knapp et al. 2002).

So far, rather few studies have looked into plant responses to changes in environmental variability (Parepa et al. 2013; Scheepens et al. 2018), specifically with respect to the timing of stress (Stone and Nicolas 1995, 1996; Prasad et al. 1999; Wang et al. 2016) or the frequency of stress (Walter et al. 2009). To our knowledge, no previous study tested for transgenerational effects of stress timing and frequency.

To address these questions and to better understand the complexity of plant responses to climatic variability (Knapp et al. 2002; Reyer et al. 2013) we carried out a two-generation experimental study with *Arabidopsis thaliana* that tested plant responses to altered timing and frequency of heat stress. To explore intraspecific variation and evolutionary potential, our study included multiple genotypes from different geographic and climatic origins. In the first generation (published in Scheepens et al. 2018) we found that the timing of heat stress had a much stronger effect on the plants than its frequency, that *A. thaliana* genotypes significantly differed in their responses to stress timing, and that this intraspecific variation correlated with the precipitation variability at the geographic origins, indicating a possible adaptive evolution of this type of phenotypic plasticity in more variable environments.

Here, we report on the results from the offspring generation where we grew plants from nine of the 11 genotypes included in the parental-generation experiment and tested on the one hand for transgenerational effects of parental stress treatments in a simple common-garden experiment, and on the other hand we subjected a subset of the offspring plants to renewed stress to test the adaptive value of transgenerational effects (reciprocal experiment). As in the parental-generation experiment, we also tested for intraspecific variation in plant responses, and we correlated this variation with climates of origin. Specifically, we asked the following questions: (1) Are there transgenerational effects of heat stress timing or frequency on the phenotypes of the offspring? (2) If yes, do transgenerational effects affect responses to current stress in an adaptive way? (3) Are there differences among *A. thaliana* genotypes in the magnitudes and/or direction of transgenerational effects? (4) If yes, does this intraspecific variation correlate with environmental conditions at the geographic origins?

Material and Methods

Parental generation experiment

The plant material used here came from a previous study (Scheepens et al. 2018) in which we tested for the direct effects of different temperature stress scenarios, varying in timing and frequency (Fig. 1), on 11 *Arabidopsis thaliana* genotypes. The 11 genotypes were selected to maximize genetic diversity and came from the "core collection" of the Versailles *Arabidopsis*

Stock Center (McKhann et al. 2004). After one week of cold-moist (4 °C) stratification, all seeds were planted into 5×5×4.5 cm pots with a 9:9:2 mixture of low-nutrient soil, regular potting soil and sterilized sand and placed in a growth chamber with 20/15°C and a 16/8 h light/dark cycle until one week after germination. For the experimental treatments, we used two identical climate chambers, one set to 20/15°C ('control chamber'), the other set to 30/25°C ('stress chamber'), both with a 16/8 light/dark cycle. A day temperature of 30°C is known to be stressful for *A. thaliana* and to reduce its fitness (Groot et al. 2017; Scheepens et al. 2018). Light conditions (230 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and air humidity (40-60%) were identical in both chambers. The experimental treatments were created by moving different subsets of plants to the stress chamber at different times and intervals. Specifically, we varied the timing and frequency of heat stress periods experienced by the plants (Fig. 1). To vary timing, we stressed plants either early in their life cycle (plants moved to stress chamber on day 8, right after the first week of seedling establishment), in the midst of most genotypes' life cycle (starting on day 26) or late in the life cycle (starting on day 44). The timing treatment was crossed with a frequency/duration treatment, where heat stress was either applied at low frequency (2 times 6 days of stress, with 6 days in between) or high frequency (4 times 3 days of stress, each time with 2 days in between). Important to note is that in all stress scenarios the plants experienced the same total time in the stress chamber and therefore also the same mean temperature during the experiment (Fig. 1). In each chamber, the spatial positions of all pots were completely randomized, and were re-randomized every week. We had eight replicate plants of each genotype in each treatment. Altogether, our parental-generation experiment included 11 genotypes × 6 treatments × 8 replicates = 528 plant individuals. The experiment ran for approximately 10 weeks. When plants began flowering, we placed their inflorescences into ARACON tubes (Betatech bvba, Gent, Belgium) to prevent cross-fertilization and collect the seeds for the next experimental generation.

Table 1. *Arabidopsis thaliana* genotypes used in this study, and their geographical coordinates and natural growing season (in months; from Scheepens et al. 2018).

Name	Country	Latitude	Longitude	Growing season
Bur-0	Ireland	54.1	-6.2	5-8
Can-0	Spain	29.21	-13.48	11-2
Ct-1	Italy	37.51	15.09	12-3
JEA	France	43.68	7.33	3-6
Mt-0	Libya	32.34	22.46	11-2
N13	Russia	61.36	34.15	6-9
Oy-0	Norway	60.39	6.19	5-8
Sha	Tajikistan	38.59	68.79	2-5
St-0	Sweden	59.34	18.06	5-8

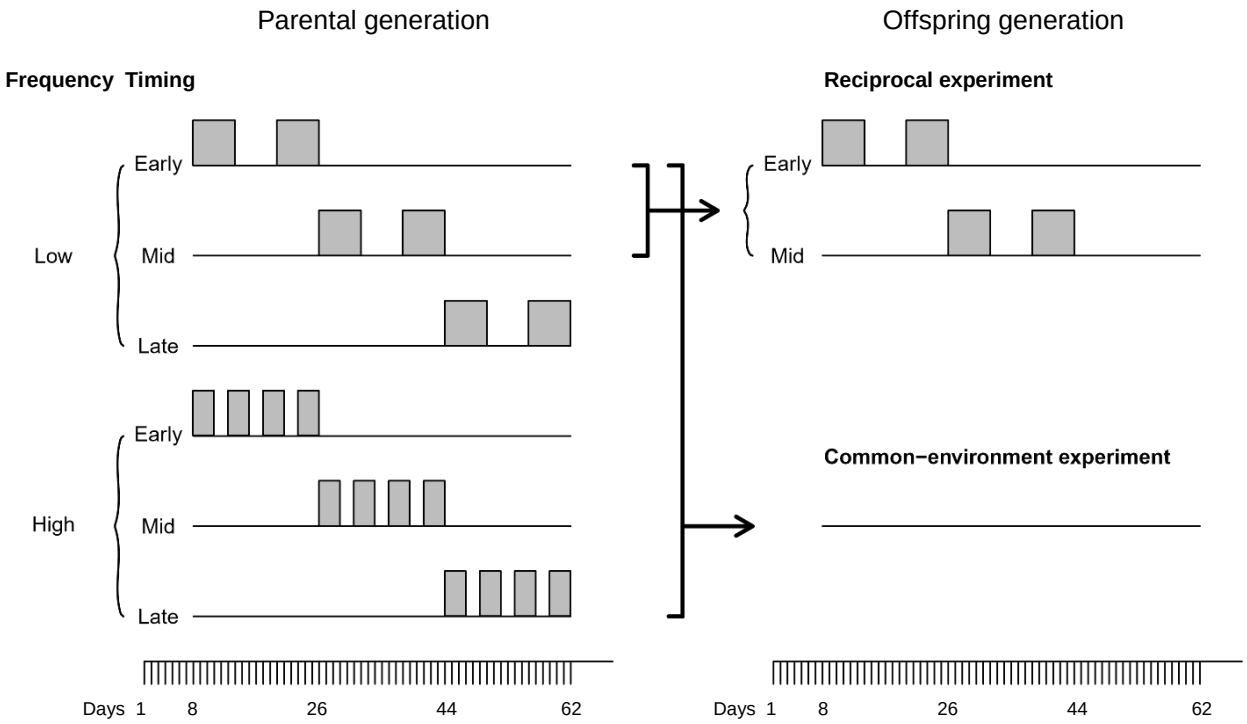

Figure 1. Experimental design of the parental-generation experiment (left) and the two offspring experiments (right) with *Arabidopsis thaliana*, with periods of 30 °C heat stress indicated in grey. In the offspring generation, plants from all parental treatments are grown in a constant control environment (common-environment experiment), and plants from two parental stress treatments are subjected to the same two treatments again (reciprocal experiment).

Offspring generation experiments

We tested for transgenerational effects in two separate experiments, (1) a simple common-environment comparison of offspring from the six parental treatments under control condition (16/8 h light/dark at 20/15 °C), and (2) a reciprocal transplant where we used offspring from only two of the parental treatments, the early and mid-term stress at low stress frequency (Fig. 1), re-created these two treatments and grew both types of offspring in both environments. We restricted the second experiment to these two treatments because they had the strongest effects in the parental generation (Scheepens et al. 2018). Since in the reciprocal experiment, plants were either grown in its "local" environment (same as parent) or a "foreign" environment that differed from the parents' environmental condition, this experiment allowed to test for adaptive transgenerational effects. In both offspring experiments we used nine of the 11 genotypes from the previous generation, because of the seed limitation in the remaining two genotypes (Table 1; Scheepens et al. 2018), and we stratified and germinated seeds as in the parental experiment. In the first experiment, we had seven replicates per genotype and maternal treatment, for a total of 9 genotypes × 6 parental environments × 7 replicates = 378 plants. In the second experiment, there were eight replicates per genotype by treatment combination, with a total of 9 genotypes × 2 maternal environments × 2 offspring environments × 8 replicates = 288 plants. In both experiments, we watered all plants regularly, and re-randomized their spatial positions every week. On day 44, right after the intermediate stress treatment in the reciprocal transplant experiment, we took leaf samples for molecular

analyses (not reported here) from 3-4 randomly selected plants from each genotype by treatment combination in each of the two experiments (i.e. from roughly half of the plants). Throughout the experiment, we recorded flowering time as the number of days from germination to when the white petals of the first flower became visible. As in the parental experiment, we placed ARACON tubes over the flowering stems to prevent outcrossing and collect seeds. Each plant was harvested one week after its fruits had started to turn yellow. We estimated plant fecundity as the number of fruits >2 mm, and we counted the number of basal shoots and lateral shoots and calculated the ratio of lateral to basal shoot number as index of plant architecture, with lower values indicating more 'shrubby' plants. After that, we separated inflorescences and rosettes, dried them at 60 °C for 72 h and weighed them, and then calculated total aboveground biomass, as well as reproductive allocation as the ratio of reproductive to total aboveground biomass.

Statistical analysis

We used linear models to test for the effects of experimental treatments, plant genotypes, and their interactions, on each of the five measured traits: flowering time, plant architecture, aboveground biomass, reproductive allocation and fecundity. For the simple common-environment experiment, the models included plant genotype, timing of parental stress, frequency of parental stress, and all possible interactions, as fixed factors. For the reciprocal experiment, the models included plant genotype, timing of parental stress, timing of offspring stress, and their interactions. Additionally, to account for possible influences of the leaf sampling, all models also included leaf sampling (yes/no) as a fixed factor. To improve the normality of residuals and homogeneity of variance, the flowering time and aboveground biomass data were log-transformed prior to the analyses.

In those cases where we discovered a significant genotype by treatment interaction, i.e. genetic variation in plasticity, in either of the two experiments, we additionally tested whether trait plasticities of genotypes were associated with (1) their fitness robustness across environments, and (2) their climates of origin. As measure of trait plasticity we used the coefficient of variation (CV) of a trait (Valladares et al. 2006) across all treatments in an experiment (common environment: six parental environments; reciprocal experiment: four combinations of parental and offspring environments). For the plasticity-fitness test we then calculated for each genotype the relative mean fitness (in terms of number of fruits) across treatments as mean fitness of genotype divided by mean fitness of best genotype (=1 for best genotype and <1 for all others), and calculated Pearson correlations between trait plasticity and relative mean fitness. For the climate-plasticity test we extracted climate data for each genotype origin from the WorldClim database (Hijmans et al. 2005), and we used on the one hand several existing bioclimatic variables that describe annual climatic variability (BIO_2 = Annual Mean Diurnal Range, BIO_3 = Isothermality, BIO_4 = Temperature Seasonality (SD), BIO_7 = Annual Temperature Range, BIO_{15} = Precipitation Seasonality (CV), and on the other hand we calculated several climate variabilities for the specific growing season (see Table 1) of each genotype: the SDs of temperature, and the CVs of precipitation, evapotranspiration and climatological water deficit. To test for relationships between climate variability of origin and the plasticity of *Arabidopsis* genotypes, we then calculated Pearson correlations between trait plasticity and the bioclimatic variables or growing-season variabilities, respectively.

All statistical analyses were done in JMP 12 (SAS Institute, Heidelberg).

Results

Common-environment experiment

In the simple common-environment experiment, we found strong genotype differences in all measured traits (Table 2), confirming that there was substantial genetic diversity in the studied *A. thaliana* genotypes. The effects of parental stress treatments were much more moderate, and were largely confined to the timing of parental heat stress: Offspring from parents which experienced early stress generally showed an increased ratio of lateral to basal shoots compared to intermediate and late stress (Fig. 2). For flowering time, the effect of stress timing depended on stress frequency (PT × PF interaction in Table 2): at high stress frequency, stress timing had an effect on flowering time, whereas at low stress frequency it did not (Fig. S1). We found significant genotype by stress timing interactions for flowering time and plant architecture (G × PT interactions in Table 2, Fig. 3), indicating genetic variation in these transgenerational responses. There were no main effects of stress frequency in any of the studied traits, and no genotype by stress frequency interactions. Only for aboveground biomass, there was a significant three-way interaction between plant genotype, parental stress timing and parental stress frequency for aboveground biomass (G × PT × PF interaction in Table 2, Fig. S2), indicating complex relationships between these three factors.

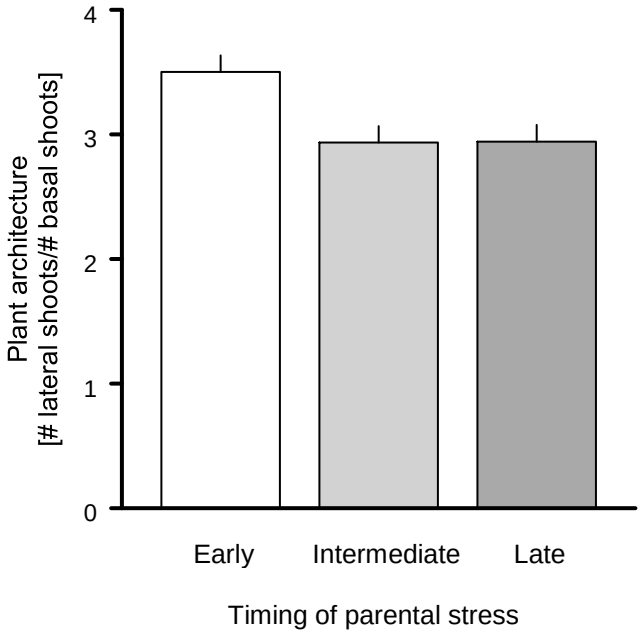

Figure 2. Effects of parental stress timing on plant architecture (number of lateral shoots / number of basal shoots) in *Arabidopsis thaliana* in the common-environment experiment.

Table 2. Results of the common-environment experiment, testing the effects of leaf sampling, genotype, parental stress timing, parental stress frequency, and their interactions, on the flowering time, plant architecture, aboveground biomass, reproductive allocation and fecundity of *Arabidopsis thaliana* offspring. Significant effects ($P < 0.05$) are in bold; df = degrees of freedom.

		Flowering time		Plant architecture		Aboveground biomass		Reproductive allocation		Fecundity	
	df	*F*-ratio	*P*-value	*F*-ratio	*P*-value	*F*-ratio	*P*-value	*F*-ratio	*P*-value	*F*-ratio	*P*-value
Leaf sampling	1	1.03	0.311	1.41	0.236	52.88	**<0.001**	20.54	**<0.001**	32.43	**<0.001**
Parental timing (PT)	2	0.85	0.429	5.96	**0.003**	0.25	0.777	1.35	0.261	1.33	0.267
Parental frequency (PF)	1	0.95	0.331	2.82	0.094	0.33	0.567	0.25	0.615	1.06	0.305
PT × PF	2	5.92	**0.003**	0.12	0.891	0.19	0.831	0.55	0.577	0.16	0.852
Genotype (G)	8	260.23	**<0.001**	99.12	**<0.001**	35.65	**<0.001**	174.37	**<0.001**	79.23	**<0.001**
G × PT	16	2.19	**0.006**	2.15	**0.007**	1.30	0.193	1.29	0.202	1.19	0.275
G × PF	8	0.40	0.920	0.54	0.829	1.22	0.287	0.88	0.536	1.30	0.242
G × PT × PF	16	0.97	0.494	1.01	0.441	1.99	**0.013**	1.47	0.109	1.10	0.353

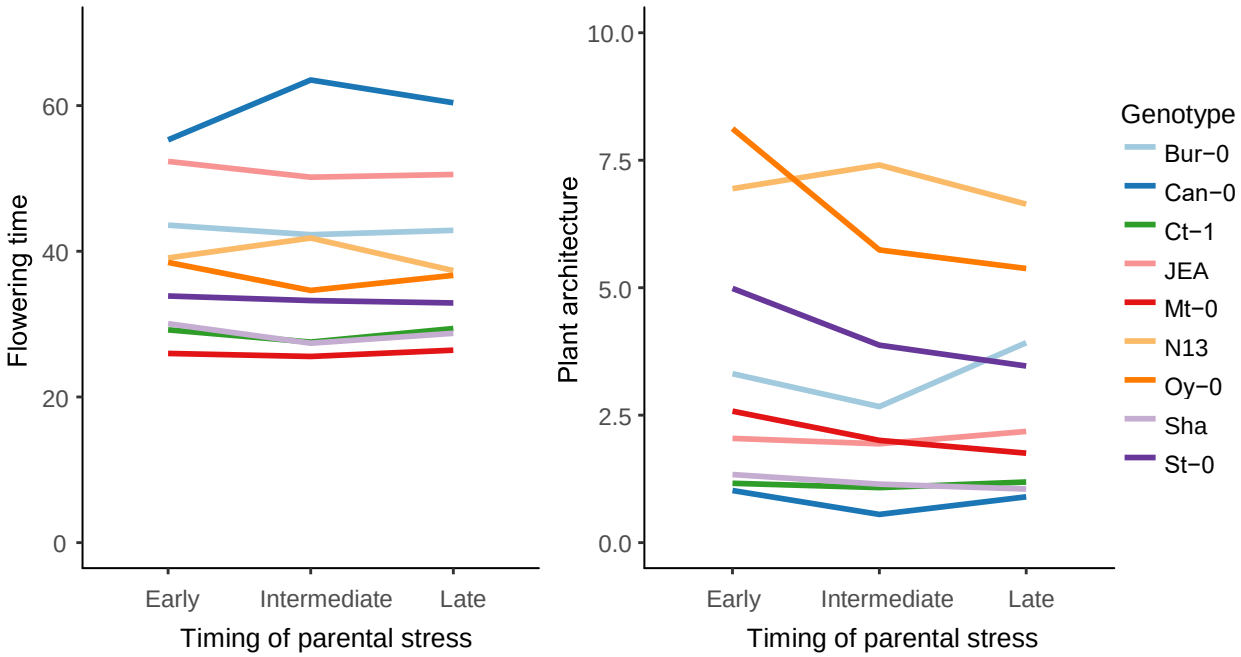

Figure 3. Genotypic variation in *Arabidopsis thaliana* in the responses of flowering time and plant architecture (# lateral shoots/# basal shoots) to different timing of parental heat stress in the common-environment experiment.

Reciprocal experiment

When offspring from early and intermediate (low-frequency) stress parents were reciprocally subjected to the same treatments, there were strong effects of offspring environment on all measured traits except for flowering time (OT main effects in Table 3), whereas the parental heat stress timing affected only the flowering time of the plants (PT main effect in Table 3), with offspring from early-stress parents flowering earlier (Fig. 4). However, a significant interaction between parental and offspring environment (PT × OT in Table 3) indicated that the expression of transgenerational effects on flowering time depended on the offspring environment: the differences between parental treatments were expressed if the offspring was subjected to early heat stress, but not if heat stress occurred later (Fig. 4).

As in the common-environment experiment, there were significant genotype differences in all of the studied traits (Table 3), and there were significant genotype by offspring environment interactions (G × OT in Table 3) in four out of the five measured traits, indicating genetic variation in (within-generation) phenotypic plasticity. In addition, we found a genotype by parental environment interaction (G × PT in Table 3), indicating genotype-specific transgenerational effects, for flowering time.

We did not find a significant parental by offspring environment interaction for plant fecundity (PT × OT in Table 3), as would have been predicted for adaptive transgenerational effects. However, there was a significant G × PT × OT interaction, indicating that these interactions are genotype-specific (Table 3, Fig. 5). We therefore tested for a significant PT × OT interaction separately for each genotype. Only in Mt-0 this interaction was significant (F = 10.38, P = 0.003), but the results did not confirm our hypothesis. In each offspring environment

the plants from the respective *other* parental environment produced more fruits than the ones from the same parental environment, suggesting rather a maladaptive transgenerational effect.

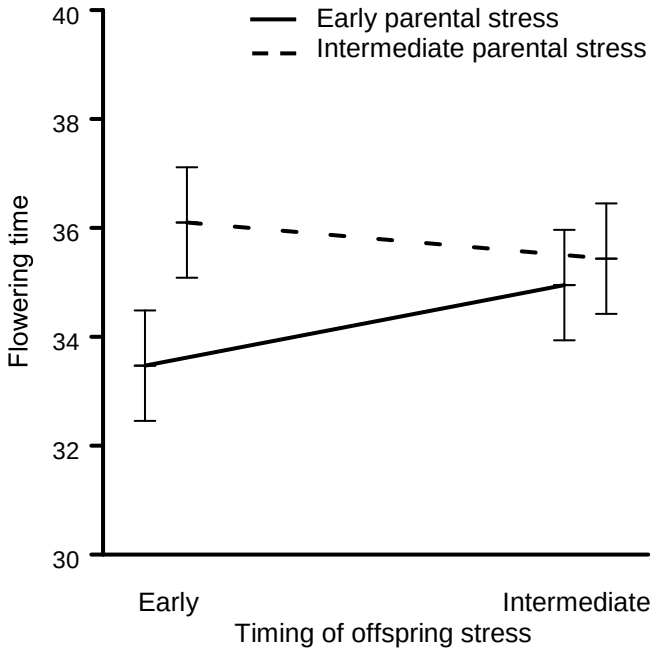

Figure 4. Effects of parental and offspring heat stress timing on flowering time in *Arabidopsis thaliana* in the reciprocal experiment.

Table 3. Results of the reciprocal experiment, testing the effects of leaf sampling, genotype, parental stress timing, offspring stress timing, and their interactions, on the flowering time, plant architecture, aboveground biomass, reproductive allocation and fecundity of *Arabidopsis thaliana* offspring. Significant effects ($P < 0.05$) are in bold; df = degrees of freedom.

		Flowering time		Plant architecture		Aboveground biomass		Reproductive allocation		Fecundity	
	df	*F*-ratio	*P*-value	*F*-ratio	*P*-value	*F*-ratio	*P*-value	*F*-ratio	*P*-value	*F*-ratio	*P*-value
Leaf sampling	1	0.00	0.960	0.14	0.707	18.38	**<0.001**	7.90	**0.005**	11.88	**0.001**
Parental timing (PT)	1	9.92	**0.002**	0.00	0.970	0.21	0.651	0.14	0.708	2.07	0.152
Offspring timing (OT)	1	0.76	0.385	8.08	**0.005**	41.77	**<0.001**	114.43	**<0.001**	17.48	**<0.001**
PT × OT	1	4.74	**0.030**	0.01	0.914	0.23	0.630	0.84	0.360	0.21	0.643
Genotype (G)	8	184.29	**< 0.001**	14.67	**<0.001**	12.13	**<0.001**	158.91	**<0.001**	57.10	**<0.001**
G × PT	8	3.50	**0.001**	0.50	0.856	0.90	0.517	1.17	0.317	0.86	0.549
G × OT	8	2.07	**0.039**	2.91	**0.004**	5.49	**<0.001**	1.59	0.128	3.97	**<0.001**
G × PT × OT	8	1.82	0.074	0.37	0.937	0.43	0.905	1.28	0.253	2.39	**0.017**

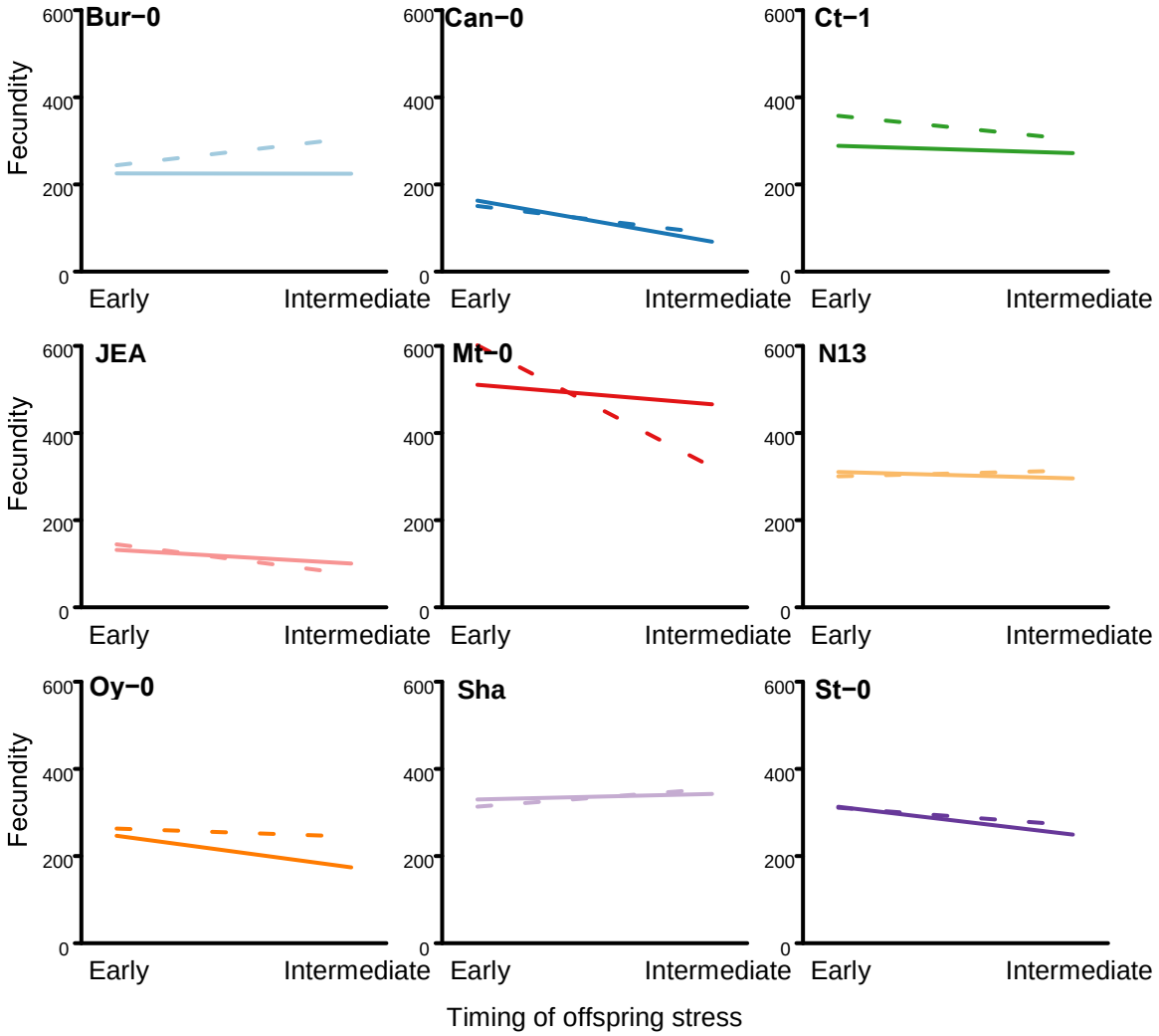

Figure 5. Genotypic variation in the effects of parental and offspring heat stress timing on fecundity in nine *Arabidopsis thaliana* genotypes in the reciprocal experiment. Solid line: early parental stress, dashed line: intermediate parental stress.

Plasticity, fitness robustness, and climate of origin

For all traits which showed significant genotype by treatment interactions (indicating genotypic variation in plasticity), we calculated genotype-level Pearson correlations between these plasticities (CVs of trait means across treatments) and (1) fitness robustness and (2) climate variables of genotype origins. In both experiments we found that the plasticity of aboveground biomass, but not that of the other traits, was significantly correlated with fitness robustness (Table S1; Fig. 6). There were no correlations at all between trait plasticity and climate of origin in the common-environment experiment (Table S2), but in the reciprocal experiment, there were several significant climate-plasticity correlations (Table S3). In particular the CV of fecundity was negatively correlated with temperature seasonality and annual temperature range, and positively correlated with isothermality (Table S3). Thus, genotypes from geographic origins with higher temperature seasonality displayed lower fecundity variation - and therefore greater fitness homeostasis - in response to different stress treatments (Fig. 7A). The CV in fecundity was also positively correlated with the seasonal CV

of evapotranspiration variability (Table S3). Moreover, we also found that the CV of aboveground biomass was positively correlated with isothermality and precipitation seasonality (Fig. 7B), and negatively correlated with latitude and with seasonal CV of climatological water deficit. Finally, the CV of plant architecture correlated negatively with the annual mean diurnal range. Despite significant genotypic variation in the response of flowering time to parental or offspring stress timing, this variation did not correlate with any of the climate variables tested.

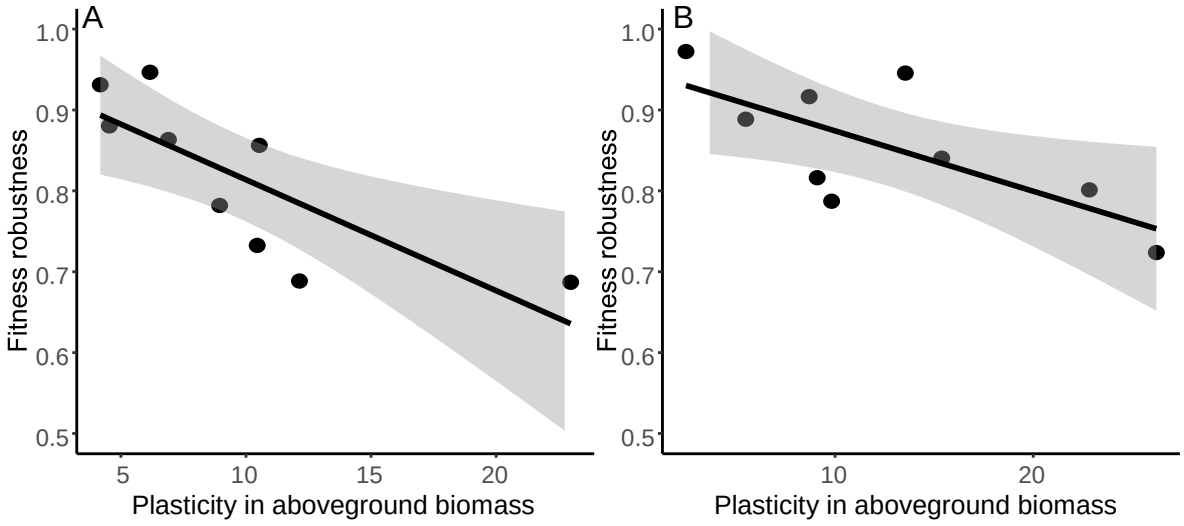

Figure 6. Relationships between fitness robustness across environments and plasticity in aboveground biomass for nine genotypes of *Arabidopsis thaliana* in the common-environment experiment (A) and in the reciprocal experiment (B).

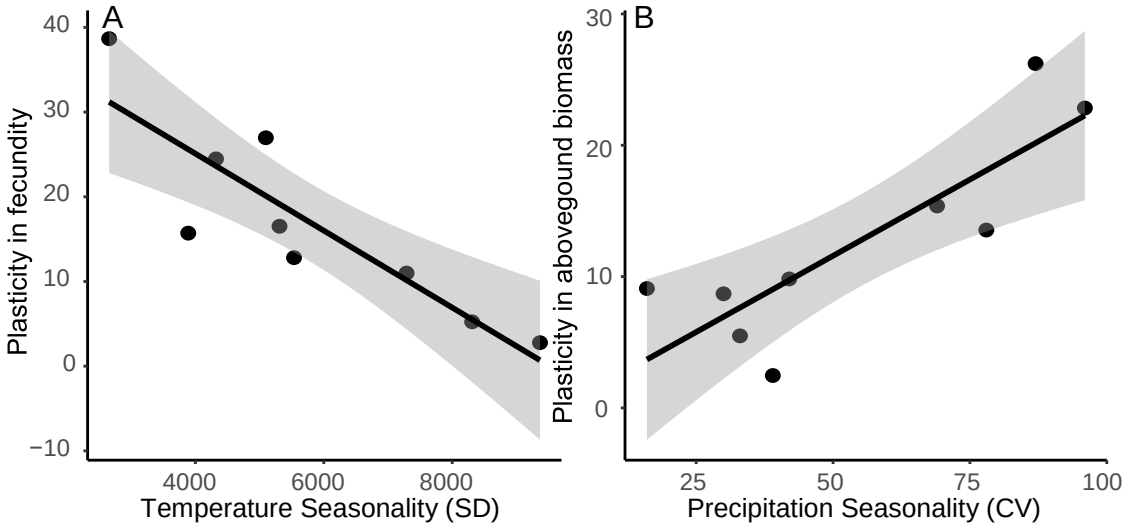

Figure 7. Relationships between trait plasticities and climates of origins for nine *Arabidopsis thaliana* genotypes in the reciprocal experiment. (A) Correlation between temperature seasonality (SD) and plasticity in fecundity. (B) Correlation between precipitation seasonality (CV) and plasticity in aboveground biomass. The plasticity values are coefficients of variation across experimental treatments.

Discussion

Changes in the temporal variability of environmental stresses are an important aspect of climate change, but we so far know little about the evolutionary consequences for plants: whether plant responses can be transgenerational, if plants harbour intraspecific variation (and thus evolutionary potential) in this respect, and how such transgenerational responses relate to environmental adaptation and fitness. Our study demonstrates that changes in the temporal patterns of heat stress can carry over to the next generation in *Arabidopsis thaliana*, and that there is substantial genotypic variation in the magnitude and direction of these transgenerational effects. Thus, changes in heat stress patterns not only affect plants directly (Scheepens et al. 2018), but also across generations. Several previous studies have reported transgenerational effects of various environmental factors (e.g. Galloway and Etterson 2007; Herman et al. 2012; Groot et al. 2017). For instance, Groot et al. (2017) subjected parental and grandparental plants of 14 *A. thaliana* genotypes to continuous heat stress and found transgenerational effects, as well as genotypic variation therein. The unique aspect of our study is that, while previous studies usually compared stressed and non-stressed plants, we only manipulated the temporal patterns of heat stress, i.e. when the stress occurred and how it was apportioned across time, whereas the total amount of stress (i.e. temperature sums) was identical in all parental environments.

Transgenerational effects of stress timing versus frequency

Overall, the timing of heat stress had much stronger transgenerational effects than its frequency, consistent with our observations in the parental plants (Scheepens et al. 2018). Variation in parental stress timing consistently affected the architecture, and, depending on the genotype and/or stress frequency, also the flowering time and biomass of offspring plants, whereas the transgenerational effects of stress frequency were only minor.

One possibility why stress frequency may play such a little role within and across generations is that plant physiological responses to heat stress may be triggered by the initial stress event, and simply remain 'switched on' afterwards, so that the number or duration of stress events does not matter, at least on the short time-scales of our experiment. A candidate mechanism for this would be heat shock proteins that plants produce to stabilize protein function (Nover et al. 2001; Sung et al. 2003; Swindell et al. 2007), and that may protect plants against subsequent heat stress events.

In contrast to stress frequency, the timing of parental heat stress influenced several traits of the plant offspring. It is generally well-established that the susceptibility of many plant traits to environmentally-induced developmental changes depends on the life stage. For instance, heat stress during floral bud development determines peg number in peanut (Prasad et al. 1999), in wheat the maximum sensitivity to heat stress for protein accumulation is during the grain filling period (Stone and Nicolas 1996), and in the herbaceous plants *Andropogon gerardii* and *Solidago canadensis* late-season heat stress causes the greatest reduction in photosynthetic productivity (Wang et al. 2016). The usual explanation for such results is that signaling pathways determining trait changes may only be active during certain developmental periods, but the precise underlying mechanisms are often unknown. Another explanation would be that no active developmental mechanisms is involved, but plants are simply more sensitive at some life stages (analogous to 'active' versus 'passive' phenotypic

plasticity; van Kleunen and Fischer 2005). In our experiment, early heat stress occurred at a small seedling stage of *A. thaliana*, whereas in the intermediate treatment the plants were already much larger and well-established. In fact, some were already bolting and/or close to flowering. It is not surprising that heat stress effects differed between these plants. However, all arguments so far, as well as the empirical studies mentioned above, are about within-generation responses to heat stress, whereas in our study we observed transgenerational effects. Thus, signaling and developmental regulation alone cannot explain our results, and there must be additional, so far unknown, physiological (Herman and Sultan 2011) and/or epigenetic (Whittle et al. 2009; Rasmann et al. 2012) mechanisms involved.

Transgenerational plasticity is not adaptive

In the reciprocal experiment we applied stress treatments to offspring plants to test their potential to adapt to stress with respect to maternal stress treatments. When offspring would have higher fitness when their parents experienced stress at the same time compared to when their parents experienced stress at another time, this would constitute an adaptive transgenerational effect. We found that responses in plant fecundity to current stress timing were dependent on parental stress timing but also varied among genotypes. In fact, the majority of the parent-offspring interactions for separate genotypes were non-significant and only the genotype Mt-0 showed a significant interaction to parental and offspring heat stress timing, although the pattern was maladaptive (i.e. offspring from parents with intermediate stress had a strong fitness loss when the offspring themselves likewise received intermediate stress). This contrasts with observations of adaptive transgenerational plasticity from previous studies (Galloway and Etterson 2007; Latzel et al. 2014). Maladaptive plasticity may be due to the expression of cryptic genetic variation expressed under stressful environments, resulting in increased trait and fitness variance which could subsequently be selected on (Ghalambor et al. 2007). However, this explanation seems unlikely for the observed maladaptive transgenerational plasticity since temperature stress was applied in all treatments. Although we cannot explain the maladaptive response in genotype Mt-0, the virtual absence of significant interactions across genotypes may reflect the lack of selective pressure for adaptive responses under unpredictable temperature stress events.

Offspring plants received early stress showed accelerated flowering when their parents experienced early stress compared when their parents experienced intermediate stress. Such advanced flowering may reflect an escape strategy (Franks 2011), which could enhance the possibility of lineage survival under continuing high temperature conditions (Wahid et al. 2007). The induction of earlier flowering by environmental stress treatments is known from previous studies (Balasubramanian et al. 2006; Franks 2011; Ibañez et al. 2017). Yet, its transgenerational plasticity and potential role in adaptation has not been commonly reported (but see Suter and Widmer 2013; Groot et al. 2017). Suter and Widmer (2013) detected accelerated flowering in *Arabidopsis thaliana* under control conditions in the fourth generation after heat exposure, but this effect disappeared in the fifth generation after two generations without stress exposure. Groot and co-workers (2017) observed earlier flowering in response to grandparental heat stress, but only in late-flowering genotypes. Our own findings indicate that exposure to high temperature at early life stage over two generations could lead to earlier flowering compared to when parental generation experienced high temperature at intermediate life stage. Although speculative, this transgenerational effect may therefore

enhance the escape strategy through early flowering. It remains an open question whether this response would be functionally adaptive under natural conditions, since it seems unlikely that heat stress events occur at the exact same time during the plant life cycle across generations.

Genotypic variation in transgenerational plasticity

Few studies have investigated intraspecific genetic diversity in transgenerational plasticity under stress conditions (Gaudet et al. 2011; Suter and Widmer 2013; Nolf et al. 2016; Groot et al. 2017) and our study provides novel evidence for it. Using nine genetically and morphologically diverse genotypes, we found significant genotype × parental treatment interactions both under control conditions and under renewed stress treatments in the offspring generation. This indicates the existence of intraspecific variation in environmentally-induced transgenerational responses in *A. thaliana*. This genotypic variation among widespread origins suggests evolutionary divergence among populations, perhaps as the result of adaptation. However, whether our results reflect the true divergence depends on the extent to which the single sampled genotype per site represents the population average (Groot et al. 2017). Populations themselves may show genetic variation in transgenerational responses, which would then form the basis for selection to act on (Endler 1986). Nevertheless, we should bear in mind that the genotypes in this study originate from a wide geographic distribution and that the available genetic variation within smaller regions or within populations is likely much more restricted (Bomblies et al. 2009).

Correlations of transgenerational plasticity with fitness robustness and climate-of-origin

We found negative correlations between fitness robustness and plasticity in aboveground biomass, but not in other traits, in the common-environment experiment and the reciprocal experiment. This is similar to the results from the parental plants (Scheepens et al. 2018) and implies that more plastic genotypes show stronger fitness variation in response to (parental and/or offspring) treatments. However, the slopes of the relationships are less steep in the offspring compared to the parental plants and fitness robustness values range from 0.69-0.95 in the common-environment experiment and from 0.72-0.97 in the reciprocal experiment compared to a range from 0.50-0.90 in the parental generation (Scheepens et al. 2018). Therefore, the offspring generation, even when under renewed stress, shows an overall improved fitness robustness, which may reflect a transgenerational adaptive response to temperature stress.

The magnitude of trait variability in response to heat stress correlated with a range of climate variables from the genotypes' geographic origins. Importantly, these relationships were only found in offspring under renewed stress treatments (reciprocal experiment) and not under stress-free conditions (common-environment experiment). This suggests that the observed correlations under renewed stress conditions (reciprocal experiment) are mainly due to the current stress environment and only partly modulated by the parental stress environment. Therefore, environmental variability at sites of origin is an important factor that could be related to plant responses to current stress and to some extent to renewed stress and could act as a selective factor leading to adaptation to environmental variability (Endler 1986).

One of the observed (negative) plasticity-environment correlations was found between plasticity in fecundity and temperature seasonality at sites of origin (Fig. 7A). Two other environmental variables reflect the same pattern, i.e. isothermality (positive correlation) and annual temperature range (negative correlation). These three environmental variables are also strongly correlated among each other (Table S4). The observation that genotypes from origins with increasing temperature seasonality show more strongly reduced plasticity in fecundity, implies that such genotypes have evolved a stronger fitness homeostasis in the face of fluctuating temperature regimes, whereas genotypes from origins with more stable temperature regimes evolved to respond more strongly to temperature stress, leading to reduced fitness in our stress experiments.

The positive relationship between plasticity in aboveground biomass and precipitation seasonality (Fig. 7B) suggests that plants from highly unpredictable precipitation environments respond strongly to temperature stress. Since biomass and fecundity are strongly positively correlated in *A. thaliana* (Clauss and Aarssen 1994), this plasticity-environment relationship seems to contrast with the above-mentioned negative correlation between plasticity in fecundity and temperature seasonality. However, precipitation seasonality and temperature seasonality do not correlate with each other (Table S4), so these plasticity-environment correlations may reflect two independent evolutionary responses to climate variability at the sites of origin.

The strongest plasticity-environment correlation was between plasticity in aboveground biomass and latitude, suggesting that plants from higher latitudes respond less to variation in temperature stress. Since increasing latitude goes along with decreasing precipitation seasonality (Table S4), the latter may be the actual environmental driver of this relationship. High precipitation seasonality at low latitudes may have selected for strong biomass responses to temperature stress, potentially reflecting escape mechanisms under periods of drought (Franks 2011).

In the parental experiment (Scheepens et al. 2018) we found positive correlations between plasticity and precipitation variability at sites of origin in four out of five traits, but we could not detect the same correlations in the offspring generation in the current study, also not when we applied renewed stress, even though transgenerational effects were still present in three out of five traits. This could imply that plant responses in the parental generation were passive and maladaptive (cf. fitness robustness) and that transgenerational effects caused the offspring generation to respond less in order to retain fitness. We did find correlations between plasticity in fecundity, plant architecture, aboveground biomass and several other climate variables in the reciprocal experiment, potentially suggesting an adaptive function of plant responses, and highlighting the relevance of environmental variability for transgenerational responses to temperature stress. A difficulty for interpreting the current results and suggesting mechanistic explanations is the discrepancy between the coarse timescale of environmental variables (year- or growing season-based) and the short life cycle of *Arabidopsis thaliana*.

Conclusions

Given that changes in temporal environmental variability are an important aspect of climate change, it is important to understand its effects on plants, both in terms of phenotypic plastic responses and of intraspecific evolutionary divergence. To our knowledge, no previous study

has shown plant transgenerational responses to temporal variability of environmental stresses, rather than their mean changes. We found ample genotypic variation in transgenerational plant responses to temporal variation in heat stress, suggesting that selection can act on it, and plasticity-environment correlations suggest an adaptation to the environmental variability of plant origins. However, we could not prove an adaptive response in the reciprocal experiment in which offspring were subjected to the same or another parental timing of temperature stress. Since signaling and developmental regulation alone cannot explain the observed transgenerational responses, we posit that physiological and/or epigenetic mechanisms are likely involved.

Acknowledgements

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Supplementary information

Table S1. Correlations between fitness robustness (relative mean fecundity) and the plasticities (CV across all treatments) of other traits across nine *Arabidopsis thaliana* genotypes. The *R*-values are Pearson correlation coefficients. Significant correlations ($P < 0.05$) are in bold.

	Flowering time		Plant architecture		Aboveground biomass	
	R	*P*-value	*R*	*P*-value	*R*	*P*-value
Common-environment	(G × PT)		(G × PT)		(G × PT × PF)	
experiment	-0.31	0.422	0.11	0.785	-0.79	**0.012**
Reciprocal experiment	(G × PT, G × OT)		(G × OT)		(G × OT)	
	-0.47	0.200	-0.15	0.702	-0.71	**0.031**

Table S2. Correlations between climates of origin and phenotypic plasticity of *Arabidopsis thaliana* genotypes in the common-environment experiment. The climate data are from the WorldClim database. The *R*-values are Pearson correlation coefficients.

Climate variables	Flowering time (G × PT)		Plant architecture (G × PT)		Aboveground biomass (G × PT × PF)	
	R	*P*-value	*R*	*P*-value	*R*	*P*-value
Growing season-based						
Temperature SD	-0.08	0.845	0.48	0.188	-0.12	0.755
Precipitation CV	0.37	0.333	0.00	0.990	-0.51	0.159
Evapotranspiration CV	0.54	0.133	0.15	0.708	0.45	0.222
Climatological Water Deficit CV	-0.19	0.624	-0.31	0.414	-0.02	0.966
Year-based						
Annual Mean Diurnal Range	-0.14	0.711	0.27	0.481	-0.56	0.113
Isothermality	0.25	0.511	0.37	0.322	-0.50	0.166
Temperature Seasonality (SD)	-0.26	0.492	-0.31	0.412	0.17	0.655
Annual Temperature Range	-0.22	0.567	-0.20	0.615	-0.01	0.983
Precipitation Seasonality (CV)	0.19	0.630	0.21	0.588	-0.39	0.300
Latitude	-0.28	0.470	-0.42	0.266	0.43	0.250

Table S3. Correlations between climates of origin and phenotypic plasticity of *Arabidopsis thaliana* genotypes in the reciprocal experiment. The climate data are from the WorldClim database. The *R*-values are Pearson correlation coefficients. Significant correlations ($P < 0.05$) are in bold.

Climate variables	Flowering time (G × PT, G × OT)		Plant architecture (G × OT)		Aboveground biomass (G × OT)		Fecundity (G × OT, G × PT × OT)	
	R	*P*-value	*R*	*P*-value	*R*	*P*-value	*R*	*P*-value
Growing season-based								
Temperature SD	0.20	0.605	-0.17	0.670	-0.20	0.609	-0.38	0.312
Precipitation CV	0.10	0.795	0.13	0.745	-0.01	0.974	0.13	0.747
Evapotranspiration CV	-0.19	0.629	0.50	0.175	0.47	0.200	0.73	**0.025**
Climatological Water Deficit CV	-0.14	0.713	0.27	0.475	-0.84	**0.005**	-0.58	0.099
Year-based								
Annual Mean Diurnal Range	0.21	0.589	-0.73	**0.027**	0.32	0.401	-0.24	0.526
Isothermality	0.45	0.225	-0.21	0.593	0.86	**0.003**	0.74	**0.022**
Temperature Seasonality (SD)	-0.37	0.325	-0.32	0.404	-0.63	0.066	-0.87	**0.002**
Annual Temperature Range	-0.28	0.469	-0.49	0.183	-0.45	0.222	-0.82	**0.007**
Precipitation Seasonality (CV)	0.05	0.889	-0.47	0.200	0.85	**0.004**	0.38	0.312
Latitude	-0.30	0.428	0.41	0.278	-0.92	**0.000**	-0.58	0.104

Table S4. Correlations between the climatic variables included in our study. The values are Pearson correlation coefficients. Significant correlations ($P < 0.05$) are in bold.

		Growing season-based					*Year-based*				
		Temperature SD	Precipitation CV	Evapotranspiration CV	Climatol. Water Deficit CV	Annual Mean Diurnal Range	Isothermality	Temperature Seasonality. (SD)	Annual Temperature Range	Precipitation Seasonality (CV)	
Growing season-based	Temperature SD	1.00									
	Precipitation CV	-0.20	1.00								
	Evapotranspiration CV	-0.56	0.13	1.00							
	Climatological Water Deficit CV	-0.07	0.47	-0.34	1.00						
Year-based	Annual Mean Diurnal Range	0.65	-0.21	-0.58	-0.43	1.00					
	Isothermality	-0.20	0.28	0.31	-0.60	0.30	1.00				
	Temperature Seasonality (SD)	0.56	-0.36	-0.60	0.30	0.30	**-0.80**	1.00			
	Annual Temperature Range	0.67	-0.35	-0.66	0.13	0.54	-0.61	**0.96**	1.00		
	Precipitation Seasonality (CV)	0.08	-0.18	0.12	**-0.85**	0.63	0.64	-0.22	0.00	1.00	
Latitude		-0.02	0.07	-0.23	**0.85**	-0.59	**-0.86**	0.48	0.24	**-0.89**	

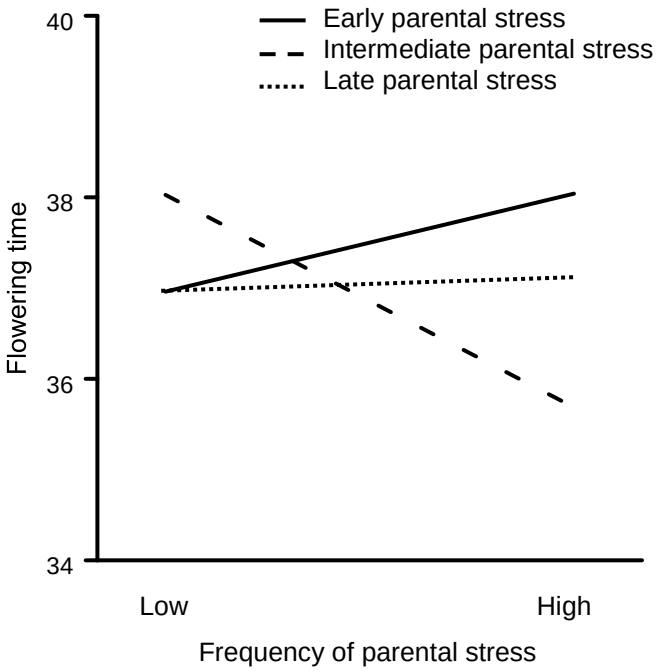

Figure S1. The effects of timing and frequency of parental heat stress on the flowering time of *Arabidopsis thaliana* in the common-environment experiment.

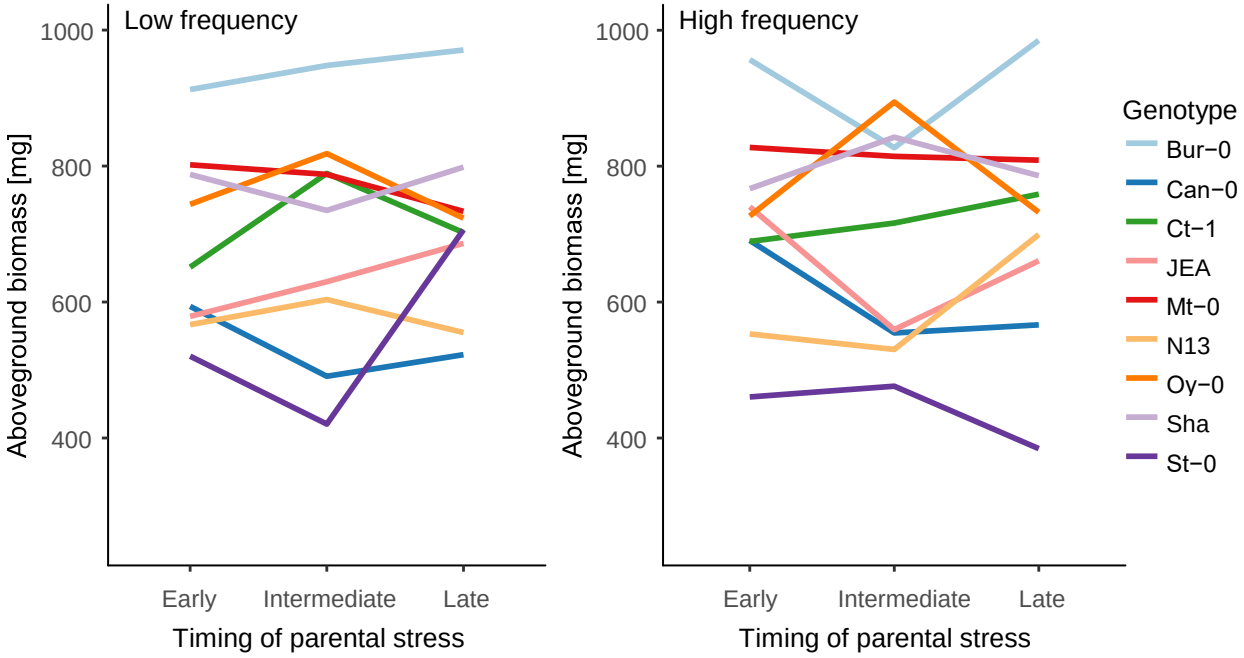

Figure S2. The three-way interaction between plant genotype, parental heat stress timing and parental heat stress frequency for aboveground biomass of *Arabidopsis thaliana* in the common-environment experiment is visualized here in two panels, the left panel showing the response of genotypes to parental stress timing under low parental stress frequency, the right panel under high parental stress frequency.

Chapter VI

General discussion

General discussion

Global change brings a series of new challenges to ecology. Besides altered means global change also includes changes in variability of abiotic factors. So far the effects of changes in variability on plants are not well studied. In plant ecological studies, there is emerging evidence that increasing environmental variability can affect plant species in their phenology, growth, reproduction, and is changing the community and ecosystems as well (e.g. Fay et al. 2000; Knapp et al. 2002; Medvigy et al. 2010). However important questions remain. For instance we do not know if the impact of environmental variability is comparable with the impact of changing environmental means, how different species and populations within those species respond to increasing environmental variability, how other environmental variables interact with the effect of environmental variability, and how this may change ecological and evolutionary processes.

This thesis attempted to address some of the remaining questions concerning how plants respond to increasing environmental variability. I worked with plant species that have a short life-span and are presumably sensitive to rapidly increasing short-term climate and environmental variations. In a suite of ecological experiments presented in this thesis, I investigated several aspects related to these questions: (1) What is the overall effect of environmental variability *per se* on plants and how it compares to the effect of environmental mean? (2) What is the relative importance of different components of environmental variability (timing and frequency of stress events)? (3) Do such effects persist across generations? (4) Are there differences among and within species in their responses to environmental variability? Below I summarize the findings from my studies, discuss how they improve our understanding of plant responses to increasing environmental variability, and identify some remaining questions.

Plant responses to environmental variability

The effect of environmental variability per se and in comparison to environmental mean - by manipulating environmental fluctuations and isolating the temporal variability of different environment variables (soil nutrients and temperature), I found that environmental variability *per se* consistently affected plants (**chapters II - V**) in several traits that include growth, phenology, reproduction, resource allocation, productivity and competitive ability. Moreover, in **chapters II & III** I found that the effect of nutrient variability is moderate compared to the effect of changing nutrient means, and can be modulated by the latter. These results confirm the findings from previous studies (e.g. Shea et al. 2004; Jentsch et al. 2007; Knapp et al. 2008) on the significance of environmental variability for plants. To identify its separate effect and the contribution it has on the overall effect of environmental changes, one needs to consider environmental variability in combination with changes in environmental means.

Relative importance of different variability components - as environmental fluctuations can have different dimensions, in **chapters VI & V** I isolated the timing and frequency of temperature fluctuations, and measured their separate effects as well as their interaction. The results showed that the two variability components have different effects and that timing rather than frequency of temperature stress has strong effect on plant performance and suggests that plant developmental stage is indeed a critical factor in determining their responses to environmental changes. Together with findings about the effect of timing of temperature stress on individual

plants (e.g. Craufurd et al. 1998; Hedhly et al. 2009), its impact on community productivity (e.g. Craine et al. 2012), our findings demonstrate the need to consider the timing of environmental changes and to understand the determining developmental factors mediating such responses.

Evidence of transgenerational responses - In **chapter V**, the second-generation *Arabidopsis thaliana* experiment provides some of the first evidence of transgenerational effects of environmental variability on plant performance. Previous studies showed that stress events which alter an environmental mean, can have maternal effects, meaning that the offspring respond to the stress their parents experienced. In my study I showed that stress events in the form of variable timing of stress, such that the mean stress experienced by all experimental plants was the same, likewise caused maternal effects. In particular, in offspring subjected to recurrent stress at early life stages, I observed advanced flowering which corresponds to studies showing that simple heat treatments induced advanced flowering responses across *A. thaliana* generations (e.g. Whittle et al. 2009; Groot et al. 2017). However I did not find any evidence that observed transgenerational effects could be adaptive (in terms of fecundity).

Inter- & intraspecific variation

In order to understand the ecological and evolutionary background of responses to environmental variability one focus of my experiments was the comparison between different species and between different populations of the same species. In **chapters II & III**, I used common annual species to investigate the variation in response to temporal nutrient variability. The results show significant species differences in the responses to changes in nutrient mean as well as nutrient variability. Thus, my study corroborates previous findings about the effects of temporal resource variation and species differences in the response to it (e.g. Novoplansky and Goldberg 2001; Liu and van Kleunen 2017). Moreover, by comparing the responses across 37 species (**chapter III**) I found that the response to nutrient variability is independent from the response to nutrient mean, indicating that they are distinct species traits.

Another part of the comparison is about variation within single species. As shown in **chapters II & IV & V**, intraspecific genetic variation is commonly present in species investigated in responses to both nutrient and temperature variability. This provides some of the early evidence of genetic variability related to responses to increasing environmental variability. Genotypic variation in such responses indicates that directional selection could in theory occur (Reznick and Ghalambor 2001; Kawecki and Ebert 2004) and, given adequate genetic variation within populations, that populations could adapt to the increased variability in environments. If such intraspecific variation is related to fitness then it provides the basis for natural selection (Strauss and Agrawal 1999). I tested for a correlation between plasticity and fitness robustness in two generations of *A. thaliana* genotypes (**chapters IV & V**), yet did not find positive correlation, so there is no evidence yet that the observed responses are adaptive. Another approach to test whether plant responses to environmental variability could be adaptive, would be to perform evolution experiments over multiple generations in which a starting population with large genotypic variation is treated under contrasting environmental variability regimes, for instance by making use of the 1001 Genomes collection of natural *A. thaliana* accessions (1001 Genomes Consortium 2016).

Among species variation and phylogeny - the development of phylogenetic analysis provides ecologists a tool for explaining differences in species responses to environmental variables from the perspective of long-term evolution. In **chapter III**, I analysed the *phylogenetic signal* in species responses to increasing environmental variability, in the case of nutrient fluctuations, and found that phylogeny can to some degree explain the variation among species. This indicates that the biotic responses/processes that are sensitive to environmental variability are to some degree conserved in closely related species. Although speculative at this point, this is possibly due to their shared genetic basis of these responses or to their overall ecological similarity and habitat preference (Harvey and Pagel 1991; Webb et al. 2002; Wiens and Graham 2005). This finding can be useful for understanding the possible consequences of increased climatic variability. Thus, if we can improve our understanding about the link between responses to environmental variability and phylogeny, in order to make predictions about the impact of changes in environmental variability one can use the phylogeny and identify the taxonomic groups that will benefit and those that will suffer from this type of changes.

Within species variation is related to climate-at-origin - populations from the same species habitating in different environments can diverge accordingly to their habitat conditions, therefore one can hypothesise that their differences in responsiveness to certain environment may reflect adaptation. In **chapters IV & V**, I tested this hypothesis with geographically widespread *A. thaliana* genotypes, and found that genotype-specific plasticity in two generations of *A. thaliana* subjecting to temperature fluctuations was correlated with climatic variability of origin, which suggests that those genotypes may have adapted to local environmental variation. However, the direction of correlation was not consistent in the two generations that I tested, with maternal generation presenting positive correlations between plasticity and climatic variability at origin (in precipitation) whereas offspring generation presenting a mixture of both positive and negative correlations in various climatic variables, so it cannot be concluded that environmental variability generally leads to increased strength of plasticity or vice versa. However, every significant relationship between trait plasticity and climatic variability on its own supports our hypothesis that similar to changes in environmental mean, changes in its variability is also potentially an important selection agent on various populations of plant species, and as a result it may drive plant adaptation under rapidly changing environments. Nevertheless, it may be too early yet to draw conclusions from my study alone, given the coarse environmental data used. Moreover, the mechanisms of the observed transgenerational effects, which may include epigenetic or physiological processes, have not been elucidated, neither for responses to mean nor for responses to variable environment.

Implications - given that different plant species and genotypes from single species co-occur in natural habitats, the differences in responses among species and genotypes can have consequences for community and population dynamics. In the case of nutrient fluctuations I found that the effect of increasing environmental variability on plant fitness reduces species differences caused by varying environmental means, and this effect is independent from changes in mean environmental conditions. Thus, I predict that environmental variability can help retain species diversity and community structure and possibly mitigate the effect of changing means. Such prediction likely go in line with the theory of fluctuation-dependent coexistence mechanisms (Chesson 2000; Roxburgh et al. 2004; Shea et al. 2004). However, community responses to the environment cannot be fully predicted based on responses of

plants grown in isolation, given that the interactions among plants, both competitive and facilitative, affect the structure of plant communities and their ecological functions (Chapin et al. 2000; Lavorel and Garnier 2002; Walther 2010) as well as their response to changing environments. To date some observations from the ecosystems, for example grassland plant biodiversity that was promoted by increased rainfall variability (Knapp et al. 2002), is in line with such prediction. More future studies are needed to test this prediction by examining how environmental mean and variability interact in affecting species coexistence and what are the consequences for various communities and ecosystems.

Outlook

My studies demonstrate the value of studying the effects of changes in environmental variability on plants, but also lead to new questions for future research. Given that each ecological study is limited by various resources, trade-offs between generality, precision, realism can be recognised; and this thesis only covers the three dimensions to some extent. Whereas several of my studies achieved a substantial degree of generality, for instance where it concerns the multi-species studies in chapters II & III, and a substantial degree of precision, for instance by considering different aspects of variability as well as multiple genotypes of a single species in chapters IV & V, all studies have been conducted under highly controlled conditions in the greenhouse or growth chamber and therefore are limited in realism (Fig. 1; adapted from Levins 1966; van Kleunen et al. 2014). To improve on generality studies in different plant systems are needed to explore the effects on longer-lived plants and plants with different life histories. To improve on realism we need to examine the ecological consequences of environmental fluctuations in mesocosms and real systems; and to examine its evolutionary consequences, we can implement long-term field studies on population dynamics. There are several ways in which precision can be improved as well: we could test the effects of environmental variability along gradients of environmental means; further decompose different variability regimes (e.g. intensity of stress events) and examine them separately as well as in combination with other aspects; and explore the extent to which plants can show plastic responses and adaptability to long-term effects. In addition, state-of-the-art molecular tools can be used to identify the genetic basis of plant responses to environmental variability *per se* and to gain a mechanistic understanding of these responses. To overcome the trade-offs identified above, we need ecologists working together within cross-disciplinary collaborations.

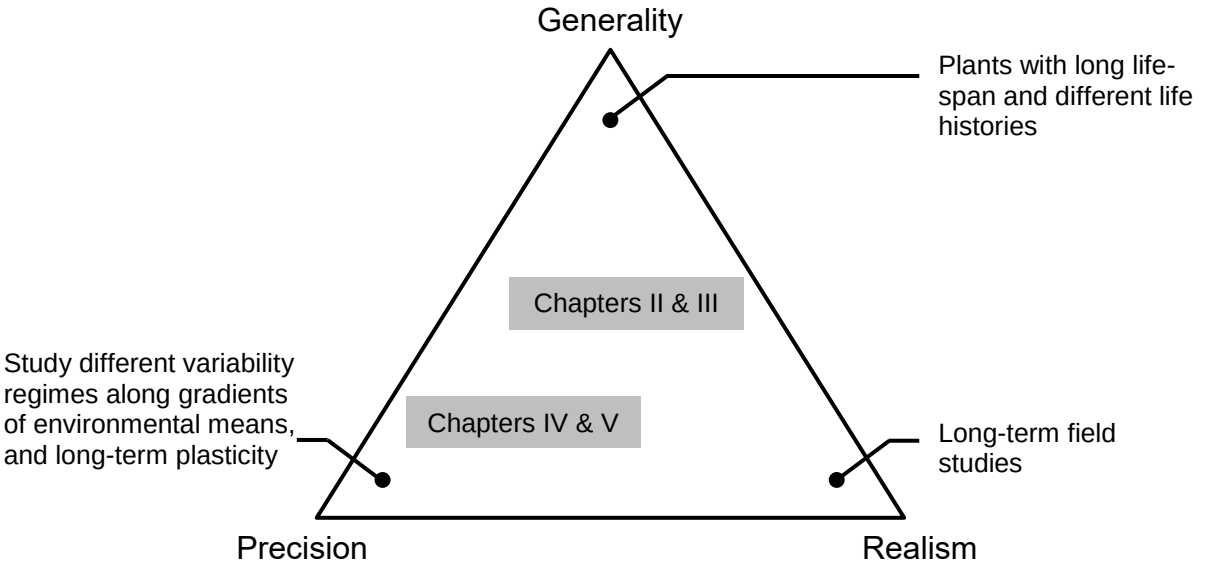

Figure 1. Given limited resources, ecological experiments are subjected to trade-offs between precision, realism and generality. In this thesis, **chapters II & III** are about two greenhouse experiments with multi-annual species and multi-populations, providing a general pattern of species variation and certain degree of genetic variation. **Chapters IV & V** are about experiments in growth chambers on one study species (*Arabidopsis thaliana*) with multiple populations and two generations, providing a demonstration of small-scale genetic variation and plasticity.

Whilst benefiting from the growing knowledge of the impacts of environmental changes on plant species, this thesis provides new insights on how plants respond to increasing environmental variability, and how their responses can be linked to past adaptation and species' evolution. Future consequences in real systems are to be expected, and I suggest experiments with long-term approaches aimed for a solid understanding of mechanisms and the ecological and evolutionary consequences.

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- Confucius

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Manuscripts in preparation

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