Evolution of plant phenotypic plasticity in response to grassland management

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Anna Lilith Shiva Kirschbaum

aus Karlsruhe

Tübingen

2020

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation: 01.04.2021
Dekan: Prof. Dr. Thilo Stehle
1. Berichterstatter: Prof. Dr. Johannes Fredericus Scheepens
2. Berichterstatterin: Prof. Dr. Katja Tielbörger

Erklärung

Zulassung als Doktorandin im Sinne von § 4 Abs. 1 der Promotionsordnung vom 24. April 2015 erfolgte am 28. Juni 2017. Diese Dissertation wurde im Sinne von §6 von Prof. Dr. Johannes Fredericus Scheepens und Prof. Dr. Oliver Bossdorf betreut.

Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass diese Dissertation von mir selbstständig – abgesehen von der Beratung und Hilfe meines Betreuers – und ohne unerlaubte Hilfsmittel erarbeitet wurde.

Andere als die angegebenen Quellen und Hilfsmittel wurden nicht benutzt und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen wurden als solche kenntlich gemacht.

Diese Dissertation wurde an keiner anderen Prüfungsbehörde vorgelegt.

Tübingen, 07.12.2020

………………………

(Anna Kirschbaum)

Dissertation eingereicht am: 07.12.2020

1. Gutachter:	Prof. Dr. Johannes Fredericus Scheepens
2. Gutachterin:	Prof. Dr. Katja Tielbörger

Acknowledgements

First and foremost, I want to express my deepest gratitude to Niek. Thank you so much for choosing me as your first PhD student and giving me the opportunity to learn from you and grow. You supported me all the way and were a great mentor also in times when you had more important things to take care off! I am especially thankful for your quick responses and support during the last few weeks of writing! It was in invaluable experience to walk my PhD journey with your guidance, your trust and your faith in my abilities. Thank you, Niek, for being a great supervisor, mentor and friend!

I also wish to thank Oliver, for letting me work in his amazing Plant Evolutionary Ecology group and for being a great second supervisor.

I am very grateful to have been able to work in such an amazing group! I had so many fun moments with all of you that made my PhD journey the best experience possible. Thanks to Anna, Bence, Brigitte, Christiane, Dario, Eva, Frank, Franziska, Ingeborg, Jun-Hee, Madalin, Martina, Robert, Sabine, Shirley, Svenja and Uta for being such supportive and fun colleagues! I especially want to thank Franziska for being the best office mate I could imagine. Thanks also to Frank and Jun-Hee for being such fun office mates, which especially during the last few weeks of writing supported me with chats and chocolate! Special thank also goes to Christiane, Eva and Sabine for all the valuable help with my experiments and all the nice lunch breaks we had together. I will miss working with all of you!

I am very thankful for the opportunity to become part of EVEREST, not only as a student but also as a representative, which gave me the chance to contribute to this wonderful network. Moreover, thanks for bringing me together with some of my best friends!

I want to thank my “Bären-Girls”, Deike, Gillian, Julia and Julia for all the amazing evenings we spent together so often. Our friendship made my years as a PhD student special and I hope we will stay close. I especially want to thank Julia G. who became a good friend during our last summer in Tübingen, who always had an open ear for me and made the “Tübinger Feierabende” unforgettable.

I also want to thank Tobi, my partner and best friend. Thank you for all your support, your humor and all the amazing adventures we share!

Last but surely not least, I want to thank my Mom for always believing in me, for supporting me in every situation and for loving me unconditionally. I owe you so much and am so grateful for being your daughter. Danke, Mama!

Table of contents

Declaration of author contributions

The thesis entitled ″Evolution of plant phenotypic plasticity in response to grassland management″ is based on the work I did during my PhD at the University of Tübingen, supervised by Prof. Dr. Johannes Fredericus Scheepens and Prof. Dr. Oliver Bossdorf. I collaborated with Dr. Madalin Parepa and with PD Dr. Günter Hoch. Chapter I – III in this thesis include three independent scientific manuscripts. Each chapter contains co-authorship and is or will be published. The contribution of the authors for each chapter is stated as following:

Chapter I
Anna Kirschbaum, Oliver Bossdorf, Madalin Parepa, JF Scheepens: *Land use plays a minor role for the evolution of plastic responses to fertilization*

Status in publication process: in preparation

JFS and OB conceived the experiment. AK, JFS and OB designed the experiment. AK conducted fieldwork and performed the experiment. AK collected data and performed data analysis with input from JFS and MP. AK wrote the manuscript with input from JFS.

Chapter II
Anna Kirschbaum, Oliver Bossdorf, JF Scheepens: *Variation in regrowth ability in relation to land-use intensity in three common grassland herbs*

Status in publication process: published in Journal of Plant Ecology, Volume 14, Issue 3, June 2021, Pages 438–450, https://doi.org/10.1093/jpe/rtab001

JFS and OB conceived the experiment. AK, JFS and OB designed the experiment. AK conducted fieldwork, performed the experiment and collected data. AK conducted data analysis with input from JFS. AK wrote the manuscript with input from JFS and OB.

Chapter III
Anna Kirschbaum, Günter Hoch, Oliver Bossdorf, JF Scheepens: *Storage of non-structural carbohydrates in Plantago lanceolata shows genetic variation among grassland populations but does not affect regrowth ability after experimental clipping*

Status in publication process: submitted to Oecologia

JFS and OB conceived the experiment. AK, JFS and OB designed the experiment. AK conducted fieldwork, performed the experiment and collected data. AK conducted laboratory work under the supervision of GH. AK wrote the manuscript with input from JFS, GH and OB.

Summary

Current environmental change, including climate change, urbanization and land-use change challenge the potential of all living organisms to adapt and survive. Plants, as sessile organisms, experience particular pressure. To cope with changing environmental conditions, plants can 1) migrate to habitats that are more suitable, 2) adapt via changes in trait means, or 3) tolerate environmental variability through phenotypic plasticity. Especially intensified grassland management, contributing to one of the major causes for global change, exerts strong selective pressure on plant populations. While the effects of land use on the evolution of trait means has already received some attention during the last decades, we know much less about the potential evolution of phenotypic plasticity in this context. As the common grassland management practices mowing, grazing and fertilization constitute recurring disturbances and thus create heterogeneous environmental conditions depending on type and intensity of land use, plants should be expected to evolve phenotypic plasticity in functional traits in order to tolerate these. Two contrasting hypotheses emerge from the idea that grassland management creates heterogeneity in environmental conditions. H1: the strength of phenotypic plasticity should increase along a gradient of increasing land-use intensity, representing increasingly heterogeneous environmental conditions (heterogenization hypothesis), and contrary H2: the strength of phenotypic plasticity should decrease along a gradient of increasing land-use intensity, as intensive land use homogenizes environmental conditions (homogenization hypothesis).

Earlier studies on the evolution of plasticity in the context of land use were often limited in their spatial extent and level of replication, and they usually only compared few contrasting environments. To advance research on land use-driven evolution of phenotypic plasticity, I investigated relationships between phenotypic plasticity and grassland management using many grassland populations along a broad gradient of land-use intensity in three regions in Germany. Specifically, I designed two common garden experiments with 58 – 68 populations of three common European grassland species - *Achillea millefolium*, *Bromus hordeaceus* and *Plantago lanceolata* – from along the land-use gradient. In one common garden experiment, I clipped half of the plants to study regrowth ability after biomass removal, representing a homeostatic response to challenging conditions. In another common garden experiment, I fertilized half of the plants to investigate opportunistic responses in biomass and nitrogen related traits to favorable conditions. With these experiments, I asked the following specific questions: i) is there genetic variation in responses to biomass removal and fertilization among plant populations? ii) is land-use intensity, especially mowing and grazing intensity, positively (heterogenization hypothesis) or negatively (homogenization hypothesis) associated with the ability to regrow after aboveground biomass removal? iii) is land-use intensity, especially fertilization intensity, positively (heterogenization hypothesis) or negatively (homogenization hypothesis) associated with the strength of opportunistic responses to a nutrient pulse? iv) is the strength of plasticity positively or negatively correlated with increasing inter-annual variation in land use? and v) does the amount of non-structural carbohydrates in the storage root of *Plantago lanceolata* influence regrowth ability after aboveground biomass removal?

The following patterns emerged: i) I found substantial genetic variation in plastic responses to biomass removal and fertilization among populations; ii) and iii) there was little evidence that land-use intensity selected for increased phenotypic plasticity, neither in regrowth ability nor in opportunistic responses to favorable conditions. However, in a few cases the strength of plastic responses was weaker in more intensively managed grasslands, thus supporting the homogenization hypothesis that increased land-use intensity selects for weaker phenotypic plasticity; iv) there was little evidence that inter-annual variation in land-use intensity selected for increased phenotypic plasticity. However, in one case, plants showed a lower regrowth ability with increasing inter-annual variation in land use; v) I found variation in the storage of non-structural carbohydrates among populations of *Plantago lanceolata*, which was however not associated with the ability to regrow after disturbance.

This thesis about intraspecific variation in phenotypic plasticity provides evidence that plastic responses can vary among grassland populations in a land-use context. This genetic variation is an important level of biodiversity as it bears potentially adaptive functions. However, I found only weak evidence for correlations with land-use intensity. Therefore, other environmental variables that still need to be identified might have driven population differentiation.

Zusammenfassung

Wir leben in einer Zeit in der sich Umweltbedingungen zunehmend schneller verändern. Dazu trägt beispielsweise der Klimawandel, die Urbanisierung und die Intensivierung der Landnutzung bei. Alle lebenden Organismen müssen sich an die sich ändernden Bedingungen anpassen, wobei vor allem Pflanzen als sessile Organismen unter besonders großem Druck stehen. Um Ihre Existenz zu sichern, können Pflanzen entweder: 1) in einen geeigneteren Lebensraum wandern, 2) sich über Veränderungen ihrer Merkmale anpassen oder 3) Umweltvariabilität durch phänotypische Plastizität tolerieren. Die intensivierte Bewirtschaftung von Grünland, die zu einer der Hauptursachen für den globalen Wandel beiträgt, übt einen starken selektiven Druck auf die ansässigen Pflanzenpopulationen aus. In den letzten Jahrzenten wurde bereits viel zu den Auswirkungen der Landnutzung auf die Evolution von phänotypischen Merkmalen geforscht. Allerdings wurde nicht untersucht, ob Landnutzung die Evolution phänotypischer Plastizität vorantreiben kann. Die gängigen Bewirtschaftungspraktiken Mahd, Beweidung und Düngung stellen wiederkehrende Störungen dar, sodass je nach Art und Intensität der Landnutzung heterogene Umweltbedingungen entstehen. Phänotypische Plastizität in funktionellen Merkmalen könnte deshalb dazu beitragen diese Umweltvariabilität zu tolerieren, dabei ist zu erwarten, dass phänotypische Plastizität unter heterogeneren Bedingungen stärker ausgeprägt ist. Daraus ergeben sich zwei gegensätzliche Hypothesen: 1) die Stärke von phänotypischer Plastizität sollte entlang eines Gradienten zunehmender Landnutzungsintensität zunehmen, da eine höhere Intensität zunehmend heterogene Umweltbedingungen schafft (Heterogenisierungshypothese), und im Gegensatz dazu 2) die Stärke von phänotypischer Plastizität sollte entlang eines Gradienten zunehmender Landnutzungsintensität abnehmen, da intensive Landnutzung die Umweltbedingungen homogenisiert (Homogenisierungshypothese).

Frühere Studien zur Evolution von phänotypischer Plastizität im Landnutzungskontext sind häufig räumlich beschränkt, verwenden wenige Replikate und vergleichen meist nur einige wenige kontrastierende Habitate. Um die Forschung zur Evolution von phänotypischer Plastizität im Zusammenhang mit Landnutzung voranzutreiben, habe ich viele Populationen entlang eines Landnutzungsgradienten in drei Regionen Deutschlands untersucht. Zu diesem Zweck habe ich zwei sogenannte "common garden" Experimente mit 58 bis 68 Populationen von drei häufigen europäischen Grünlandarten – Gewöhnliche Schafgarbe (*Achillea millefolium*), Weiche Trespe (*Bromus hordeaceus*) und Spitzwegerich (*Plantago lanceolata*) – durchgeführt. Diese Populationen liegen entlang eines Gradienten von extensiver bis sehr intensiver Landnutzung, was geringer Beweidung ohne Düngung bis zu mehrmaliger Mahd mit hohem Düngeeinsatz entspricht. Um zu untersuchen wie die Fähigkeit nachzuwachsen von der Intensität der Landnutzung der vergangenen Jahre abhängt, habe ich in einem Experiment bei der Hälfte der Pflanzen die oberirdische Biomasse entfernt. In dem anderen Experiment habe ich die Hälfte der Pflanzen gedüngt, um zu untersuchen, wie sogenannte opportunistische Reaktionen, wie beispielsweise das Wachstum, als Reaktion auf günstige Umweltbedingungen, von der Intensität der Landnutzung der vergangenen Jahre abhängt.

Mit diesen zwei Experimenten wollte ich konkret die folgenden fünf Fragen beantworten: i) Gibt es zwischen den untersuchten Populationen genetische Variation für phänotypische Plastizität? ii) Korreliert die Fähigkeit zum Nachwachsen nach dem Entfernen von Biomasse positiv (Heterogenisierungshypothese) oder negativ (Homogenisierungs-hypothese) mit der Landnutzungsintensität und insbesondere mit der Mahd- und Beweidungsintensität? iii) Korreliert die Stärke der opportunistischen Reaktionen auf einen Düngepuls positiv (Heterogenisierungshypothese) oder negativ (Homogenisierungs-hypothese) mit der Landnutzungsintensität und insbesondere der Düngeintensität? iv) Korreliert die Stärke der plastischen Reaktionen positiv oder negativ mit zunehmender Variation von Landnutzung über die Jahre hinweg (zwischenjährliche Variation)? und v) Beeinflusst die Menge an nicht-strukturellen Kohlenhydraten in der Speicherwurzel von Spitzwegerich die Fähigkeit zum Nachwachsen nach der Entfernung von Biomasse?

Basierend auf den Ergebnissen aus den Experimente können die Fragen wie folgt beantwortet werden: i) zwischen den unterschiedlich bewirtschafteten Grünlandpopulationen gibt es eine hohe genetische Diversität in den Reaktionen auf das Entfernen von Biomasse und Düngung; ii) und iii) in den meisten Fällen hängt weder die Stärke der Nachwachsfähigkeit noch der opportunistischen Reaktionen mit der Landnutzungsintensität zusammen. In einigen wenigen Fällen jedoch war die Stärke der plastischen Reaktionen in intensiv bewirtschafteten Grünländern schwächer. Dies unterstützt die Homogenisierungshypothese, welche besagt, dass phänotypische Plastizität unter erhöhter Landnutzungsintensität schwächer ausgeprägt ist; iv) in den meisten Fällen hängt die Stärke der plastischen Reaktionen nicht mit der zwischenjährlichen Variation der Landnutzungsintensität ab. In einem Fall jedoch zeigten die Pflanzen eine geringere Fähigkeit zum Nachwachsen, wenn sich die Landnutzung über die Jahre hinweg stark verändert; v) zwischen den Spitzwegerich Populationen gibt es genetische Diversität in der Speicherung von nicht-strukturellen Kohlenhydraten. Allerdings hängt die Fähigkeit zum Nachwachsen nicht von der Menge der gespeicherten nicht-strukturellen Kohlenhydrate ab.

Diese Arbeit über intraspezifische Variation von phänotypischer Plastizität im Landnutzungskontext liefert Hinweise darauf, dass die plastischen Reaktionen zwischen unterschiedlich bewirtschafteten Grünländern variieren können. Diese genetische Variation ist ein wichtiger Bestandteil der Biodiversität, da aus ihr adaptive Veränderungen entstehen können. Jedoch war der Zusammenhang zwischen phänotypischer Plastizität und Landnutzung eher gering. Diese Ergebnisse deuten darauf hin, dass andere potentielle Umweltvariablen die Differenzierung der Populationen vorangetrieben haben.

General Introduction

Plant adaptations to land use

Since the beginning of the Anthropocene around 12000 – 15000 years ago (Agricultural Revolution), when humans became the most important factor in changing biological, geological and atmospheric processes, environmental conditions change rapidly (Jump and Peñuelas 2005; Steffen *et al.* 2015; Waters *et al.* 2016). All organisms need to track these changes and adjust in order to survive. Plants as sessile organisms are especially vulnerable to drastic changes in environmental conditions and need to adjust quickly. They can either (1) track favorable conditions through migration (Davis and Shaw 2001; Jump and Peñuelas 2005), (2) adapt to the novel conditions through changes in trait means (Davis and Shaw 2001; Jump and Peñuelas 2005; Hoffmann and Sgrò 2011) or (3) tolerate environmental variability (Bradshaw 1965; Strauss and Agrawal 1999; Munns and Tester 2008).

Under current rates of unprecedented global change, plants face a wide array of environmental challenges, such as climate change or urbanization (Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services 2019). However, the most important direct driver of global change is land-use change, which has the largest relative impact on terrestrial and freshwater ecosystems (Díaz *et al.* 2019). The most significant human alteration of the environment most rapidly accelerated with the so-called "green revolution" in the 1950s and 1960s, when high-yielding cultivars, the processes of mechanization and irrigation, and the unrestricted use of fertilizers and pesticides were introduced into agricultural practices (Matson 1997; Foley *et al.* 2005). The globally increasing demand of food led to an increase in fertilizer use of about 500 % - 700 % between 1960 and 2000 (Matson 1997; Foley *et al.* 2005; Steffen *et al.* 2015). Additionally, looking specifically at grasslands for animal husbandry, about 25 % of ice-free land is used as pastures for grazing and meadows for haymaking (Foley *et al.* 2005; Díaz *et al.* 2019). These numbers illustrate how far-reaching the effects of ever-increasing land-use change have been so far and likely will be in the future.

Common land-use practices in grasslands, namely grazing, mowing and fertilization, thus influence a big portion of plants worldwide and challenge the potential of adaptation and survival of many plant species. As such, evolution of locally adapted populations in a grassland context can be expected, as land use differs among populations, and has indeed been found in empirical studies. For instance, classical studies on the evolution of plant traits and intraspecific adaptation to land use in grasslands found population differentiation in response to soil and fertilization (Snaydon and Davies 1972; Davies and Snaydon 1974) as well as to grazing and mowing pressure (Warwick and Briggs 1979; Van Tienderen and van der Toorn 1991). Additionally, also more recent studies confirmed the evolution of dwarf morphology and prostrate growth forms (Warwick and Briggs 1979; Völler *et al.* 2017), as well as tolerance to damage (Louault *et al.* 2005; Díaz *et al.* 2007) and phenological shifts (Reisch and Poschlod 2009; Völler *et al.* 2017) as adaptations to grazing and mowing. Increasing plant stature on the other hand, has been found as response to increased fertilization (Snaydon and Davies 1972; Davies and Snaydon 1974). In summary, genetically based changes in trait means in response to land use are widespread and represent a suitable solution to adapt to environmental changes. However, when these changes occur at a faster rate hampering the ability to adapt trait means, it might be more advantageous for plants to tolerate the changing environmental conditions. As such, the evolution of phenotypic plasticity, allowing for a broader habitat niche and short-term adjustments to changing environmental conditions (Jump and Peñuelas 2005), could additionally facilitate plant survival under current global change.

Phenotypic plasticity

Phenotypic plasticity describes the ability of a single genotype to alter its phenotypic expression depending on environmental conditions (Bradshaw 1965; Sultan 2000; Pigliucci 2005). This means that one genotype produces different phenotypes in different environments (environment-dependent phenotypic expression) (Sultan 2003). In early ecological studies, the effect of the environment on the expression of a phenotype was long seen as a nuisance without ecological and evolutionary relevance (Bradshaw 1965; Pigliucci 2005). However, in the last decades the importance of phenotypic plasticity in contributing to the ability of plants to tolerate abrupt environmental changes or such that are too rapid to be met with evolutionary processes was acknowledged (Bradshaw 1965; Schlichting 1986; West-Eberhard 1989; Pigliucci 2005). Phenotypic plasticity itself is a genetically controlled trait and as such, it is subject to evolution by natural selection (Bradshaw 1965; Sultan 1987; West-Eberhard 1989; Pigliucci 2005). Thus, adaptive plasticity should exist in nature (Pigliucci 2005). For plasticity to be considered adaptive, it should allow the plant to maintain its function and even increase plant performance across environments (Sultan 1995; Alpert and Simms 2002). However, plasticity does not only include adaptive plant responses but also inevitable reactions to environmental limitations such as resource shortage (Sultan 1995, 2000). It is important to note that type and strength of plasticity is not a property of the whole genotype but specific to individual traits and environments (Bradshaw 1965; Sultan 1995). This means, for instance, that a specific trait could show plastic responses toward nutrient availability but not toward water availability but a different trait could react to water instead (Pigliucci 2006). The so-called reaction norm of a genotype, which depicts the environment-dependent phenotypic expression, is a common demonstration of plasticity, where the slope of the reaction norm in the environment-phenotype space describes the strength of plasticity (Schlichting 1986; Sultan 1995). If different genotypes from the same population show non-parallel responses of the same trait towards the same environmental variable (genotype-by-environment interaction), genetically based variation for plasticity exists (Schlichting 1986; Sultan 1995). Selection can than act upon this variation if different environments select for different trait values (Schlichting and Levin 1990; Via *et al.* 1995; Van Kleunen and Fischer 2005; Pigliucci 2005; Matesanz *et al.* 2012). As such, intraspecific phenotypic plasticity is a source of intraspecific variation and represents an important level of biodiversity.

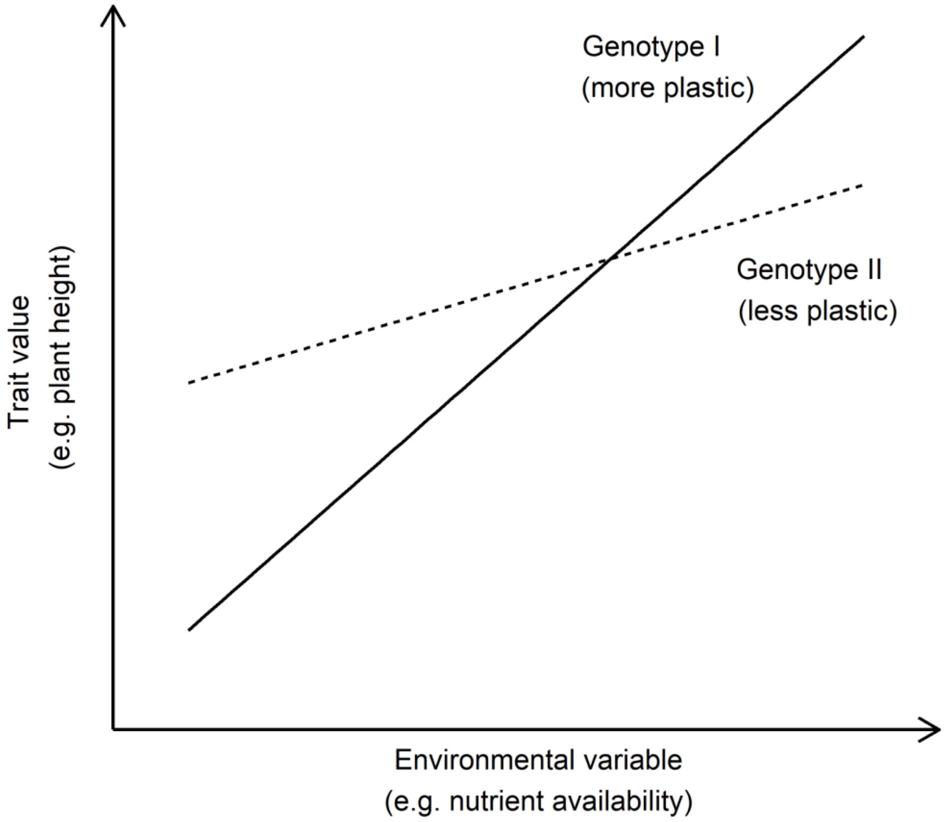


Figure 1: Reaction norms of two genotypes differing in the strength of phenotypic plasticity in a certain trait (e.g. plant height) along an environmental gradient (e.g. nutrient availability). Adapted from Pigliucci (2005).

It is especially useful for a plant to be plastic when environmental conditions are variable over space and time (Van Kleunen and Fischer 2005). Thus, adaptive plasticity should evolve and be selected for under heterogeneous environmental conditions (Weinig 2000; Donohue *et al.* 2001; Van Kleunen and Fischer 2001; Gianoli and González-Teuber 2005; Scheiner 2013; Lázaro-Nogal *et al.* 2015). In such a scenario, a genotype with a narrow ecological breadth determined by its mean trait values would probably go extinct, whereas the plastic genotype would be able to withstand the changing conditions (Sultan 2000). In contrast, in a homogeneous environment plasticity should not evolve or should even be lost, assuming that certain costs and limits might constrain the evolution of plasticity (Tufto 2000; Relyea and Morin 2002; Valladares *et al*. 2007; Auld *et al.* 2010).

Generally, the evolutionary implications of phenotypic plasticity are diverse and could influence patterns of genetic diversification and macroevolution (Sultan 2000). First, phenotypic plasticity contributes to the ability of individuals and even whole species to withstand sudden environmental changes and buffers against environmental variability, thus reducing the risk of extinction (Robinson and Dukas 1999; Sultan 2000). Second, highly plastic genotypes that produce appropriate phenotypes in different local environments might hamper adaptive diversification by weakening selection on the genotype (Sultan 2000; Sultan and Spencer 2002). Third, in contrast, plasticity could instead also facilitate adaptive divergence either through genetic assimilation (Pigliucci and Murren 2003; Pigliucci 2005, 2006) or through buffering short-term environmental variability and thereby allowing for more time to evolve fixed adaptations (Pigliucci 2001, 2005; West-Eberhard 2003).

A common approach to study both local adaptation and phenotypic plasticity of natural populations is the transplantation of plants from different populations into common gardens. This is a way to investigate the effect of different environments on the expression of the phenotype. However, instead of replicating several common gardens with different environmental conditions it is also possible to manipulate the environmental cues of interest in only one common garden (Sultan 2000). If genotypes differ in their response to this manipulation a genotype-by-environment interaction and thus genetic variation for plasticity exists. It is though important that the genetic material for comparisons between gardens or treatments is as similar as possible. Ideally, genetically identical individuals (clones) are used (Sultan 1995, 2000). However, other set-ups that minimize genetic variability between individuals in different environments/treatments (e.g. a half-sib design) are also possible. To assess the plastic response, manipulated individuals are compared to closely related control individuals.

Plastic responses to land use

The idea that common land-use practices, such as grazing, mowing and fertilization could select for phenotypic plasticity arises from the fact that these create recurring disturbances both within one year (intra-annual) and among several years (inter-annual) creating spatiotemporal heterogeneity (Suzuki 2008). To understand how land use creates heterogeneous conditions within one year, the individual aspects of each practice need to be dissected. Mowing represents a distinct event during plant growth, depriving a plant of almost all aboveground biomass, which represents a dramatic loss for plants, especially when it hits the plants during their reproductive phase. To ensure reproduction, plants need to compensate via regrowth. Because farmers have virtually unlimited access to artificial fertilizer nowadays, grassland productivity has increased dramatically. Thus, grasslands can even be cut up to four times a year (Vogt *et al.* 2019), repeatedly changing environmental conditions, such as light or competition (Gibson *et al.* 2011). As this represents temporal heterogeneous conditions within one year, mowing might select for the ability to compensate via regrowth, constituting a plastic response. Common grazing practices on the other hand create both temporal and spatial environmental heterogeneity. Temporal heterogeneous conditions are created by confining the grazing period or changing grazing pressure (type and number of livestock) within one year. Additionally, spatial heterogeneity results from selective grazing, trampling and patchy dung deposition such that within a site conditions vary over small scales (Adler *et al.* 2001; Bloor and Pottier 2014). The practice of grazing might thus challenge the plants among others with repeated aboveground biomass removal, increased light availability and localized nutrient enrichment, which might promote the evolution of plastic responses. The practice of fertilization also creates temporal and spatial heterogeneity. Temporal heterogeneity results from discrete events of fertilizer application at several occasions a year, repeatedly changing soil nutrient conditions. The manner of applying the fertilizer, mostly uneven large-scale spraying, especially when using organic fertilizers such as manure or slurry, additionally creates localized patches of enriched nutrients and thus spatial environmental heterogeneity. Thus, the practice of fertilization might promote the evolution of phenotypic plasticity, too. On top of all this, farmers might change their grassland management from year to year to meet current developments on their farms. This might include transforming a pasture into a meadow or vice versa, or adapting fertilizer use and the number of mowing events (Vogt *et al.* 2019). On an inter-annual timescale, such variations might create additional heterogeneity in environmental conditions that plants must cope with. It would thus be expected that increasing land-use intensity, meaning higher mowing frequency, increased livestock density and grazing period as well as increased amounts of fertilizer, representing increasingly heterogeneous environmental conditions, should select for stronger phenotypic plasticity. This is further referred to as the heterogenization hypothesis. However, increasing land-use intensity might as well have the potential to homogenize environmental conditions, such that intermediate and low intensities represent more fluctuating and thus heterogeneous conditions (Benton *et al.* 2003). High mowing intensity for example causes biotic homogenization (Olden *et al.* 2004; Gossner *et al.* 2016) as well as sward uniformity increasing light availability (Benton *et al.* 2003; Gibson *et al.* 2011). High fertilizer input might as well cause biotic homogenization (Olden *et al.* 2004; Gossner *et al.* 2016) and leads to nutrient accumulation in the soil (Vitousek *et al.* 2009), buffering variation in intra- and inter-annual nutrient input. Additionally, increases in duration and intensity of grazing lead to decreased structural heterogeneity (Adler *et al.* 2001; Benton *et al.* 2003) as well as reduced vegetation height further homogenizing light conditions (Fuller and Gough 1999). Thus, in contrast to the heterogenization hypothesis, it could also be expected that under increasing land-use intensity, homogenizing environmental conditions, the strength of plasticity would decrease. This is further referred to as the homogenization hypothesis. Concluding, depending on type and intensity, common land-use practices on grasslands generate recurring disturbances that lead to temporal and spatial environmental heterogeneity in growing conditions and as illustrated above, plants might evolve phenotypic plasticity to cope with these repeatedly changing conditions.

In general, plastic responses can be broadly categorized into opportunistic responses to unpredictable and favorable conditions or into robustness, or homeostatic responses under stressful conditions (Richards *et al.* 2006). In a land-use context, fertilization might initiate opportunistic responses to capitalize rapidly on local increases in nutrient supply. This might include root morphological or physiological plastic responses to increase nutrient uptake, increased photosynthetic capacity or N use efficiency to get the most out of the temporally increased nutrient availability (Chapin 1980; Aerts and Chapin 1999; Grime and Mackey 2002; Hodge 2004). Translated to a plasticity context, such an opportunistic response in underlying morphological or physiological traits would relate to a steeply increasing reaction norm of fitness parameters under increased nutrient availability (Richards *et al.* 2006). Tolerance, which is the ability to maintain relatively constant fitness under stressful conditions such as recurring grazing or mowing, constitutes a homeostatic response (Simms 2000; Barton 2013). One key trait of grazing- and/or mowing-tolerant plants is the ability to regrow after biomass removal and thus to partially or fully compensate the lost biomass (Strauss and Agrawal 1999; Huhta *et al.* 2003). Expressing this in a plasticity context, a fully damage-tolerant plant, plastically adjusting underlying morphological and physiological traits, would show a flat reaction norm of fitness across different damage intensities (Rejmánek 2000; Simms 2000; Richards *et al.* 2006).

As land-use type and intensity vary among grasslands, even on a very small scale between neighboring pieces of land, the type and strength of plastic responses should evolve accordingly at this small spatial scale and manifest in intraspecific population differentiation (Silvertown *et al.* 2006), as long as counteracting forces such as gene flow among populations do not constrain differentiation. In the analysis of a common garden experiment with a manipulation with populations from differently managed grasslands this would appear as a population-by-environment (i.e. treatment) interaction and would represent inter-population genetic variation for the response towards the manipulation, i.e. genetic variation for plasticity (Schlichting 1986). This would indicate that populations evolved differently strong responses towards common land-use practices and could contribute to local adaptation. The regression of the strength of the response, i.e. a plasticity index, on land-use intensity would then show the strength and direction of an association between plasticity and land use.

As suggested above, depending on type and intensity, land use creates heterogeneous environmental conditions and should thus select for phenotypic plasticity (Briggs 2009). Despite the straightforward predictions, the evolution of plasticity in a land-use context received comparably less attention than the evolution of trait means. Additionally, most studies remained limited in their level of replication and spatial extent and only tested for intra-annual temporal variation in land use, neglecting the possibility that inter-annual variation in land use could also drive the evolution of plasticity. Highlighting some studies that looked into the evolution of plasticity in a land use context, a study on *Senecio vulgaris* showed that high fertilizer input in an agricultural habitat selected for plants with a stronger increase of leaf area and reproductive biomass after nutrient addition compared to plants from a ruderal site (Leiss and Müller-Schärer 2001). In the context of biomass removal, *Persicaria longiseta* plants from one grazed population in Nara Park responded similarly to clipping as plants from two ungrazed populations, suggesting no evolution of increased regrowth ability under a grazing regime (Suzuki 2008). In contrast, the ability to regrow after clipping was greater in *Schizachyrium scoparium* plants from three grazed sites compared to three ungrazed sites, suggesting the evolution of adaptive plasticity under a scenario of recurring biomass removal (Carman and Briske 1985). Underlying morphological or physiological traits contributing to regrowth ability might for instance include an increased number of tillers or branches (Lennartsson *et al.* 1998; Strauss and Agrawal 1999; Agrawal 2000; Huhta *et al.* 2003), enhanced photosynthetic capacity (McNaughton 1979; Sultan *et al.* 1998; Strauss and Agrawal 1999) or the use of carbon storage compounds, so called non-structural carbohydrates (Oesterheld and McNaughton 1988; Strauss and Agrawal 1999; Turner *et al.* 2006; Palacio *et al.* 2012). Concluding, as these and many other studies only compare a few environments, remain limited in their spatial extent and number of replicates, and as no clear conclusions can be drawn from these ambiguous results, the effects of land use on the evolution of plasticity need to be investigated more thoroughly. Ideally, the relationship between the strength of plasticity and land use should be studied along a whole gradient of land-use intensity, to infer the actual shape of the reaction norm and intra- as well as inter-annual variation in land use should be considered.

Non-structural carbohydrates

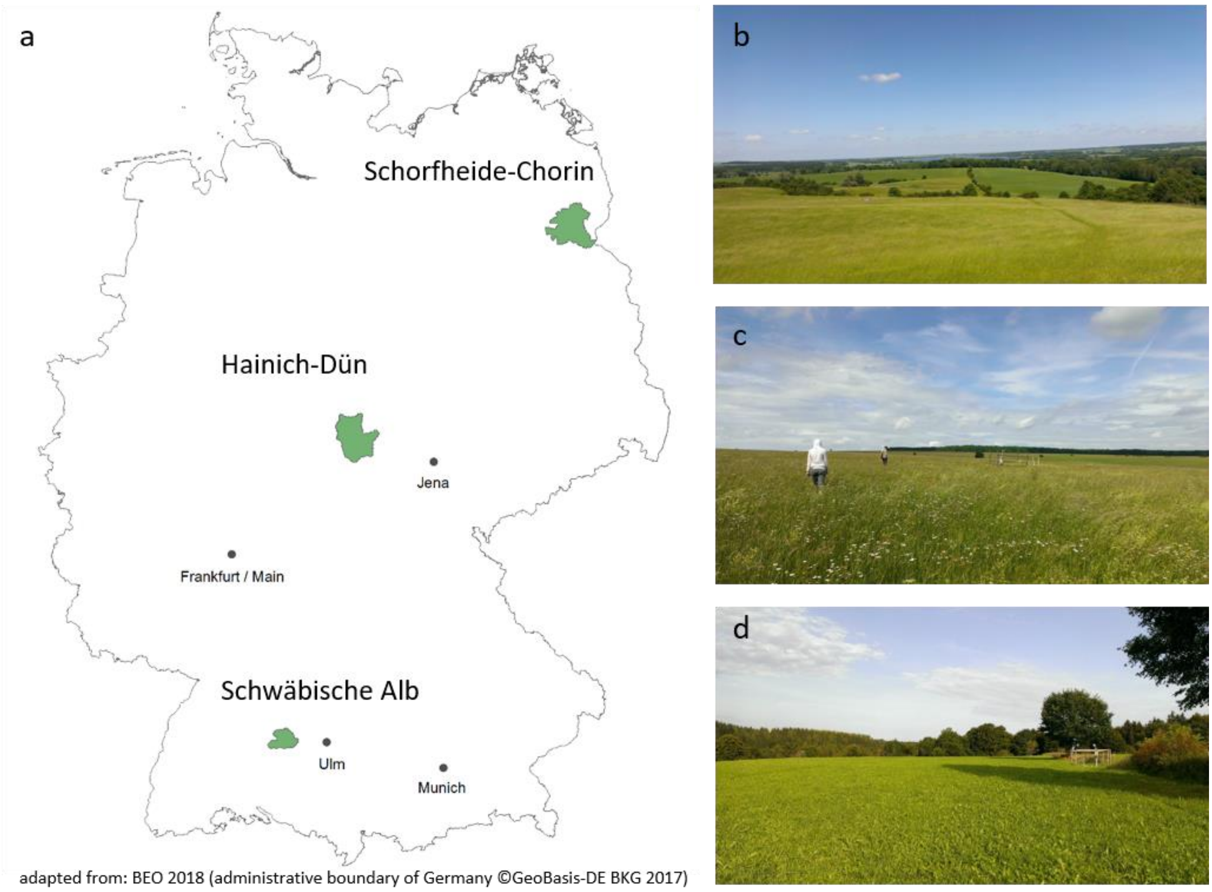
As mentioned in the previous paragraph, plants may have evolved different capabilities to regrow after aboveground biomass removal. This might be facilitated by the storage of non-structural carbohydrates (NSCs) that are composed of low molecular weight sugars, such as fructose, sucrose and glucose, and starch (for some herbs and grasses additionally fructans) (Chapin *et al.* 1990; Martínez-Vilalta *et al.* 2016; Landhäusser *et al.* 2018). Carbon that is assimilated through photosynthesis during the day is mainly used as substrate for metabolism and structural growth, but a small fraction is stored in the form of NSCs in stems, leaves or roots but also in specialized organs (Janeček and Klimešová 2014; Martínez-Vilalta *et al.* 2016). NSCs can be mobilized in times of low carbon availability to support for example metabolic functions during the night or a variety of other plant functions in stressful situations, when carbon assimilation does not meet demand (Chapin *et al.* 1990; Smith and Stitt 2007; Dietze *et al.* 2014; Martínez-Vilalta *et al.* 2016). Hence, NSC mobilization is suggested to play a role during flowering and seed production (Horibata *et al.* 2007), during spring regrowth (Heilmeier *et al.* 1986) or to facilitate regrowth after aboveground biomass removal (Greub and Wedin 1971; Richards and Caldwell 1985; Li *et al.* 2002; Carpenter *et al.* 2008). When a plant loses most of its aboveground biomass, for example as a result of grazing or mowing, it is deprived of the ability to photosynthesize and regrowth of photosynthetically active tissue is prioritized (Richards and Caldwell 1985; Visser *et al.* 1997; Morvan-Bertrand *et al.* 1999). Indeed, it has been shown that NSCs are mobilized from storage tissue after aboveground biomass loss and are translocated to newly sprouting shoots to facilitate regrowth (Hodgkinson 1969; Danckwerts and Gordon 1987; Morvan-Bertrand *et al.* 1999; Schnyder and de Visser 1999). The ability to mobilize stored NSCs would thus be especially advantageous in a grassland context that is characterized by recurring biomass removal.

As illustrated above, land use exerts strong selective pressure on a plethora of genetic and phenotypic traits. Additionally, when NSCs are important for regrowth ability after biomass loss, it could be expected that the ability to store and mobilize carbon is under selection in a land-use context, that is characterized by recurring grazing and mowing. As land-use type and intensity differ among populations, differentiation among populations in the amount of stored NSCs before the onset of grassland management, speed or strength of NSC mobilization after grazing or mowing and replenishment of NSC reserves in autumn is expected. It already has been shown that plants can adapt their levels of NSC storage to severity of biomass loss (Palacio *et al.* 2012; Benot *et al.* 2019). For instance, NSC levels of heavily grazed grassland plants were higher than in moderately grazed ones (Benot *et al.* 2019), highlighting the selective potential of grassland management on NSC storage. However, the question how different levels of NSCs before disturbance affect regrowth potential still needs further exploration, as no consensus exists about the question if higher NSC levels also increase regrowth after disturbance (Davies 1965; Richards and Caldwell 1985; Hogg and Lieffers 1991).

Study system

Biodiversity Exploratories

I studied the effects of land-use intensity on the evolution of plasticity in grassland species within the Biodiversity Exploratories, a large-scale and long-term research project studying the relationships between land use, biodiversity and ecosystem functions (https://www.biodiversity-exploratories.de). The three exemplary research sites, the so-called Exploratories – the UNESCO Biosphere Reserve Schorfheide-Chorin, the Hainich National Park and surrounding areas, and the UNESCO Biosphere Area Schwäbische Alb – are located on a north-south transect within Germany with approximately 300 km distance between each other (Fischer *et al.* 2010). Each Exploratory features 50 grassland sites, measuring 50×50 m, that are located a few hundred meters up to 40 km apart (Fischer *et al.* 2010). Farmers manage these sites, as they would usually do and provide information on type and intensity of land use in yearly inventories (Vogt *et al.* 2019). Intensity of mowing is characterized as the number of cuts per year; grazing intensity is calculated as livestock units per hectare, multiplied with the grazing period and weighted by livestock type (cattle, sheep, horse, goat); intensity of fertilization is given as amount of nitrogen applied per hectare (Blüthgen *et al.* 2012). For all my analyses, I used a long-term measure of each land-use type to integrate land-use history and inter-annual variation. Land-use intensity was calculated as the global mean for all three Exploratories overall for the years from 2006 to 2016 according to Blüthgen *et al*. (2012) based on information from the land owners on mowing, grazing and fertilization (Vogt et al. 2019) using the LUI calculation tool (Ostrowski *et al.* 2020) implemented in BExIS (http://doi.org/10.17616/R32P9Q). The fact that all sites are managed as usual and the broad range of land-use intensity among sites, ranging from a very extensive use with only light grazing to a highly intensive use with up to 170 kg of nitrogen applied per hectare and with up to 4 cuts per year, make the Biodiversity Exploratories an excellent framework for studying local adaptation and population differentiation in response to common grassland management.



adapted from: BEO 2018 (administrative boundary of Germany ©GeoBasis-DE BKG 2017)

Figure 2: **a**) Location of the three Exploratories: the UNESCO Biosphere Reserve Schorfheide-Chorin, the Hainich National Park and surrounding areas, and the UNESCO Biosphere Area Schwäbische Alb. **b**) Meadow in the UNESCO Biosphere Reserve Schorfheide-Chorin; grazing intensity = 0, mowing intensity = 1.61, fertilization intensity = 0. **c**) Pasture in the Hainich National Park; grazing intensity = 0.43, mowing intensity = 0.08, fertilization intensity = 0. **d**) Mown pasture in the UNESCO Biosphere Area Schwäbische Alb: grazing intensity = 1.04, mowing intensity = 2.04, fertilization intensity = 2.97. Intensity of land use is averaged across 11 years (2006-2016).

Study species

For my thesis, I selected *Achillea millefolium*, *Bromus hordeaceus* and *Plantago lanceolata* as study species. This decision was based on frequent occurrence of the species on the sites of the Biodiversity Exploratories, easy seed sampling and cultivation as well as previous knowledge on phenotypic plasticity.

Achillea millefolium, commonly known as common yarrow, is an erect herbaceous perennial grassland plant native to temperate regions, especially Eurasia and North America (Warwick and Black 1982; CABI 2020). It is self-incompatible and pollinated by a great array of insects (Foster 1988). *Achillea millefolium* has lanceolate leaves that form a basal rosette and alternate along one to several stems that grow between 0.2 – 1 m in height. White to pink ray and disk flowers form the flat-topped inflorescence cluster (Warwick and Black 1982). *Achillea millefolium* reproduces sexually via achene-like seeds and vegetatively via rhizomes (Grainger and Turkington 2013). Common yarrow is well-known to be plastic towards shade in several traits such as leaf greenness, leaf dry matter content or leaf area (Bourdôt 1984; Dostál *et al.* 2016). Additionally, plastic responses in *A. millefolium* towards fertilization were found for plant height, leaf greenness (Dostál *et al.* 2016) and total biomass (Drenovsky *et al.* 2012). Because of its demonstrated plasticity and the association with fertilization, *Achillea millefolium* is well-suited for investigating the selective potential of land use on plasticity.

Bromus hordeaceus, commonly named soft brome, is an annual grass species native to European meadows and annual grasslands (CABI 2020). *Bromus hordeaceus* is mostly self-pollinating and predominantly autogamous (Ainouche *et al.* 1999; Völler *et al.* 2013). Soft brome is entirely pubescent and grows 10-100 cm tall erect or ascending culms, that often grow in tufts (Clayton *et al.* 2006). Concerning land use, grazing and mowing have been found to select for marked population differentiation in height and flowering phenology in *B. hordeaceus* (Völler *et al.* 2013). Furthermore, two perennial conspecific species (*Bromus erectus* and *Bromus inermis*) have been shown to be highly plastic towards fertilization, shading and waterlogging (Dostál *et al.* 2016). Thus, *Bromus hordeaceus* is a promising candidate for investigating the effects of land use on the evolution of phenotypic plasticity.

Plantago lanceolata, also known as ribwort plantain, is a rosette-forming perennial herbaceous grassland species, native to Eurasia but now globally distributed (Cavers *et al.* 1980). Deeply furrowed flowering stalks growing between 10 cm and 40 cm in height from a rosette of lanceolate leaves carry ovoid inflorescences with many small flowers (CABI 2020). *Plantago lanceolata* is a wind-pollinated obligate outcrosser and thus shows high gene flow among populations (Gáspár *et al.* 2019). The taproot of ribwort plantain is well developed and functions as a storage organ for NSCs (Janeček *et al.* 2011). Ribwort plantain is generally known to be plastic in many morphological and physiological traits (Kuiper 1984; Kuiper and Bos 1992). Moreover, in a land-use context, *Plantago lanceolata* showed local adaptation (Van Tienderen and van der Toorn 1991) and population differentiation in 17 phenotypic traits (Wolff and Van Delden 1987). Additionally, Dostál et al. (2016) showed that *Plantago lanceolata* is especially plastic to fertilization and Warwick and Briggs (1979) found this species to be plastic in response to different mowing/grazing regimes. Because of its demonstrated plasticity towards common land-use practices and the ability to store NSCs, *Plantago lanceolata* is well suited for investigating the selective potential of grassland management on the evolution of plasticity in general and especially on the evolution of regrowth ability facilitated through the mobilization of NSCs after grazing or mowing.

Objectives

The selective potential of common land-use practices, such as grazing, mowing and fertilization, has been demonstrated in many systems and species. However, how land use affects the evolution of intraspecific phenotypic plasticity has gained much less attention. Furthermore, the studies investigating plasticity in response to land use often remained limited in their level of replication, mostly worked on a restricted spatial area and compared only few contrasting environments. My aim was to overcome these limitations by studying the evolution of intraspecific phenotypic plasticity within the framework of the Biodiversity Exploratories, making use of a big number of populations along a gradient of land-use intensities. In two common garden experiments with *Achillea millefolium*, *Bromus hordeaceus* and *Plantago lanceolata* collected from the Biodiversity Exploratories, I mimicked natural fertilization through nitrogen addition in a greenhouse experiment and grazing or mowing through a clipping treatment in an outdoor common garden.

In **Chapter I** of my thesis, I analyzed the intraspecific opportunistic responses of the three grassland plants towards nutrient addition in two functional traits and aboveground biomass. Leaf chlorophyll content and leaf N content are known to increase with increasing nutrient input, particularly nitrogen. As central part of the photosynthetic machinery, chlorophyll influences plant performance, i.e. biomass. I fertilized half of my plants in the experiment after 10 weeks of growth and calculated a plasticity index for every trait as the log response ratio between fertilized and unfertilized plants. In a first step, I analyzed if populations differed in their response to the fertilization, which would suggest intraspecific interpopulation genetic variation for plasticity. In a second step, I related the plasticity indices to the strength of fertilization, grazing and mowing and their inter-annual temporal variation.

In **Chapter II** of this thesis, I investigated the intraspecific homeostatic response of regrowth ability of the three focal grassland plants after a clipping treatment. I clipped half of the plants after 7 weeks of growth and let them regrow for 16 weeks until the end of the growing season. At the end of the experiment, I calculated an index of plasticity of regrowth as the log response ratio of biomass between clipped and unclipped plants. In a first step, I analyzed if populations differed in their ability to regrow after clipping, which would suggest intraspecific interpopulation genetic variation for plasticity. In a second step, I related the plasticity indices to the strength of grazing and mowing and their inter-annual temporal variation.

In **Chapter III** of my thesis, I analyzed the storage of non-structural carbohydrates (NSCs) in the taproot of *Plantago lanceolata* and its influence on regrowth ability. I photometrically measured the content of glucose and starch in an additional, unclipped batch of *P. lanceolata* plants from the outdoor common garden experiment (Chapter II). The measurement took place at the same time as the plants from the clipping experiment were clipped. I analyzed if populations differed in their levels of NSC and if this population differentiation is related to land use. Additionally, I was able to relate levels of NSC at the time of clipping to regrowth ability and plant performance.

Chapter I

Land use plays a minor role for the evolution of plastic responses to fertilization

Anna Kirschbaum, Oliver Bossdorf, Madalin Parepa, JF Scheepens

Abstract

Aims

Management of grasslands, characterized by grazing, mowing and fertilization, exerts strong selection on the resident plant populations. Evolutionary changes in the means of ecologically important traits in response to these land-use practices have been shown in many previous studies. However, how land use influences the evolution of phenotypic plasticity to cope with spatial and temporal variation caused by grassland management has received much less attention. In this study, we investigated the relationship between opportunistic plastic responses to fertilization and land-use intensity as well as temporal variation in land use.

Methods

In a common garden experiment, we tested if plants from more intensively managed grasslands evolved stronger or weaker opportunistic responses in biomass, leaf nitrogen and leaf chlorophyll content in response to a fertilizer pulse than plants from less intensively managed grasslands. For this purpose, we used seed material from three common European grassland species from 58-68 populations along a gradient of land-use intensity. We grew two offspring from 5-7 seed families per population in the greenhouse and applied fertilizer to half of the plants after 10 weeks of growth.

Important findings

In two out of three species (*Achillea millefolium*, *Bromus hordeaceus*), plant responses to fertilization differed significantly among populations in almost all measured traits, suggesting interpopulation variation for plasticity. While variation in the response to fertilization of *A. millefolium* populations was related to fertilization and mowing intensity, the responses of the other two species showed no association with land use. Interestingly, we detected a trade-off between nutrient conservation and acquisition in terms of leaf chlorophyll content in *Bromus hordeaceus* and *Plantago lanceolata*, which suggests that populations responding opportunistically to fertilization suffer more under nutrient scarcity. Concluding, although variation in phenotypic plasticity was detected in some species, the potential selection pressures shaping this variation remain to a large extent unclear.

Keywords: environmental heterogeneity, fertilization, grazing, inter-annual temporal variation, intraspecific variation, mowing, phenotypic plasticity

Introduction

Land-use change is a major global change driver (IPCC 2019). To increase productivity of currently around one quarter of the Earth's land surface that is used as meadows and pastures for haymaking or livestock grazing (Ramankutty and Foley 1999; Foley *et al.* 2011), global fertilizer use has increased 500 % - 700 % between 1960 and 2000 (Matson 1997; Tilman 2001; Foley *et al.* 2005, 2011). Under these unprecedented rates of land-use change, especially the increase in N deposition, plant traits such as flowering phenology, leaf N content, photosynthetic rate and biomass allocation are expected to be affected (Matesanz *et al.* 2010) and to adapt either through changes in mean trait values or in the expression of phenotypic plasticity. Recurring disturbances in managed grasslands, such as fertilization, grazing and mowing, create heterogeneous growing conditions over space and time either within one growing season (intra-annual) or across growing seasons when management practices change between years (inter-annual). Fertilization for example, creates heterogeneity in soil nutrient conditions in space (localized patches of high and low nutrient availability) and time (several fertilization events during a year) (Vogt *et al.* 2019). However, when fertilization intensity is high, soil nutrient conditions might not change much and become rather homogeneous in space and time such that low or intermediate fertilization intensities represent a more fluctuating environment in terms of nutrient availability. Grazing creates heterogeneous conditions in space through selective grazing, trampling and patchy dung deposition (Adler *et al.* 2001). Mowing on the other hand, creates heterogeneity in environmental conditions in time (several mowing events per year) but homogenizes conditions in space. However, high mowing intensity might homogenize environmental conditions such as light or species composition also in time (Socher *et al.* 2013; Gossner *et al.* 2016). Additional temporal heterogeneity is created when management practices – type and/or intensity – change from year to year, which represents temporal variation in management (Allan *et al.* 2014; Kirschbaum *et al.* 2021). Hence, depending on type and intensity, common grassland practices create spatially and temporally heterogeneous growing conditions, which challenge the potential of plants to adapt and survive. As former studies mostly focused on the evolution of mean traits in response to land use (Warwick and Briggs 1979; Díaz *et al.* 2007; Reisch and Poschlod 2009, 2011; Völler *et al.* 2017), we aim at investigating whether phenotypic plasticity in functional traits evolved along land-use gradients.

As sessile organisms, one mechanism of plants to adapt to heterogeneous environmental conditions is through phenotypic plasticity, which describes the ability of a single genotype to change phenotypic traits depending on biotic and abiotic conditions (Bradshaw 1965; Schlichting and Levin 1986). As a genetically controlled trait itself (Schlichting and Levin 1986; Pigliucci 2005; Scheiner 2013), plasticity of a specific trait might evolve if it increases fitness across environments (Matesanz *et al.* 2010) and if genetic variation for plasticity exists within a population (genotype-by-environment interaction) (Pigliucci 2005). It has been shown in theoretical as well as in empirical studies that plasticity is especially advantageous under heterogeneous environmental conditions, where it should thus evolve (Sultan 1987; Stuefer 1996; Balaguer *et al.* 2001; Alpert and Simms 2002; Gianoli 2004; Gianoli and González-Teuber 2005; Valladares *et al.* 2007; Scheiner 2013). In contrast, under homogeneous conditions, if being plastic bears certain costs, plasticity should not evolve or should even be lost (Van Kleunen and Fischer 2005). Therefore, we propose that under increasing land-use intensity, phenotypic plasticity in plant functional traits should evolve either to become stronger, if high land-use intensity increases heterogeneity in environmental conditions or to become weaker, if high land-use intensity rather homogenizes environmental conditions. Under rising N deposition in managed grasslands, this might be especially true for traits responsible for resource allocation and resource use, as plasticity in underlying morphological and physiological traits could increase plant performance and ultimately plant fitness.

Resource allocation in plants depends on nutrient availability in the soil and root uptake capacity, which a plant can modify through several morphological and physiological changes in structures related to resource acquisition, use and conservation. These changes are often very plastic and either improve nutrient accessibility or enhance resource use (Chapin 1980; Hutchings and de Kroon 1994; Aerts and Chapin 1999; Hodge 2004). Morphologically plastic adaptations for example include changes in root morphology (e.g. total root length, root diameter, root elongation, lateral branching), root:shoot ratio, specific leaf area or biomass allocation (Hutchings and de Kroon 1994; Grime and Mackey 2002; Hodge 2004). As these adaptations involve the building of new tissue or the replacement of existing tissue, they are characterized as slow response – high-cost solutions (Grime and Mackey 2002). In contrast, physiologically plastic responses, such as changes in leaf C:N ratio, leaf N content or photosynthetic capacity constitute rapid changes at the subcellular level that are characterized as fast response – low-cost solutions (Grime and Mackey 2002; Hodge 2004).

On the one hand, plastic responses to nutrient availability can be characterized as opportunistic responses to favorable conditions, such as after a fertilization event, which is analogous to the master-of-some scenario to increase fitness (Richards *et al.* 2006). Following Diaz and colleagues (2004), this strategy of opportunistic responses can also be described as the nutrient acquisitive type, which is typically represented by fast-growing species from highly fertile habitats with a high nutrient uptake capacity (Reich 2014) and a high degree of (morphological) plasticity (Chapin 1980; Aerts and Chapin 1999; Hodge 2004). On the other hand, homeostatic responses of plants under stressful conditions, such as when nutrients become limiting, are analogous to the jack-of-all-trades scenario (Richards *et al.* 2006) and ensure fitness homeostasis. This strategy, also known as the conservative type, prevails in plants from low-fertility habitats which evolved a conservative nutrient-use strategy (Díaz *et al.* 2004; Reich 2014) and generally show a lower degree of (physiological) plasticity (Chapin 1980; Aerts and Chapin 1999; Hodge 2004). Plant strategy specialization along the acquisition conservation trade-off axis is a global phenomenon (Reich *et al.* 1997; Díaz *et al.* 2004, 2016; Wright *et al.* 2004). However, intraspecific differences in nutrient-use strategies, for example as adaption to environmental gradients, gained more attention only recently (Niinemets 2015; Isaac *et al.* 2017; Martin *et al.* 2017; Sartori *et al.* 2019).

The type as well as the strength of plasticity in response to habitat fertility have mostly been studied at the interspecific level, and some studies confirm (Crick and Grime 1987; Dong *et al.* 1996; Grassein *et al.* 2010) whereas others falsify (Boot and Mensink 1990; Van de Vijver *et al.* 1993; Fransen *et al.* 1999; Bowsher *et al.* 2016) the above-mentioned strategies. However, studies at the intraspecific level that investigate if plastic responses differ between populations that evolved in habitats differing in soil fertility remain scarce. A classic intraspecific study is from the famous Park Grass Experiment, in which *Anthoxanthum odoratum* plants that were fertilized with phosphorus for the last 120 years were shown to have a stronger response to P-fertilization in dry matter production than plants historically not fertilized (Davies and Snaydon 1974). Contrastingly, a study on *Prunella vulgaris* showed that low N accessions have a higher degree of plasticity in N use efficiency under increased nutrient availability compared to high N accessions (Wedlich *et al.* 2016). However, most of the studies investigating intraspecific differences in patterns of plasticity remain limited in their replication as well as spatial extent and only compared a few contrasting environments. However, by sampling many populations along a land-use gradient, we are able to extract a true signal of the effects of fertilization intensity on the evolution of plasticity.

In this study, we investigated the degree of intraspecific plasticity in populations of three common grassland plants along a land-use gradient. On the one hand, we expected that the degree of morphological and physiological plasticity in traits such as biomass, leaf N content and leaf chlorophyll content would increase with increasing intensity of fertilization, grazing and mowing, representing increasingly heterogeneous environmental conditions (heterogenization hypothesis). This would also be in accordance with the notion that plasticity should be higher in high fertility soils than in low fertility soils. However, on the other hand it is also conceivable that the degree of morphological and physiological plasticity in the afore mentioned traits could decrease with increasing intensity of fertilization, grazing and mowing, representing increasingly homogeneous environmental conditions (homogenization hypothesis). Additionally, we tested the relationship between opportunistic nutrient uptake and homeostatic nutrient conservation, to identify potential intraspecific trade-offs along the acquisition conservation trade-off axis (Martin *et al.* 2017; Sartori *et al.* 2019). For this purpose, we performed a common garden experiment with a fertilization treatment on plants originating from 58 populations of *Achillea millefolium*, 69 populations of *Bromus hordeaceus* and 63 populations of *P. lanceolata* sampled along a land-use gradient ranging from extensive to very intensive management. In particular, we asked the following questions: 1) Is there population differentiation in plant responses to increased nutrient availability (i.e. variation in phenotypic plasticity)? Is this population differentiation related to 2) intra-annual variation in land-use intensity and its underlying components or to 3) inter-annual variation in land use and its underlying components? Additionally, we asked 4) if there is a trade-off between opportunistic nutrient uptake after fertilization and nutrient conservation under nutrient limitation in leaf chlorophyll content and whether such a trade-off is related to land-use intensity.

Material and Methods

Study system

We conducted our study within the framework of the Biodiversity Exploratories, a large-scale and long-term research project in Germany investigating the relationships between land use, biodiversity of different taxa and ecosystem functioning (Fischer *et al.* 2010); https://www.biodiversity-exploratories.de/en). The so called "Exploratories" – the UNESCO Biosphere Reserve Schorfheide-Chorin, the Hainich National Park and surrounding areas and the UNESCO Biosphere Area Schwäbische Alb – span a north-south transect in Germany and comprise 50 grassland plots each that are managed by local farmers. The grassland plots, each with an area of 50 × 50 m, are located at distances of a few hundred meters to 30-40 km (mean distance 13.4 km) within each Exploratory and cover a land-use gradient from extensive to very intensive management. Type (grazing, mowing, fertilization) and intensity of land use is recorded yearly based on information from the land owners (Vogt *et al.* 2019) and is calculated as follows: grazing intensity is estimated as livestock units per hectare, multiplied with the grazing period and weighted by livestock type (cattle, sheep, horse, goat); mowing intensity is given as the number of cuts per year; fertilization intensity is quantified as amount of nitrogen (kg) applied per hectare (Blüthgen *et al.* 2012). According to Blüthgen *et al*. (2012), long-term land-use intensity of the three factors fertilization (F_{mean}), grazing (G_{mean}) and mowing (M_{mean}) was calculated as the global mean over all three regions from 2006 – 2016, using the LUI calculation tool (Ostrowski *et al.* 2020) implemented in BExIS (http://doi.org/10.17616/R32P9Q). Additionally, we calculated an index of inter-annual land-use variation for fertilization intensity (F_{var}), grazing intensity (G_{var}) and mowing intensity (M_{var}) as the coefficient of variation of each factor from 2006-2016. F_{mean}, G_{mean} and M_{mean} served as a proxy of intra-annual heterogeneity of environmental conditions, whereas F_{var}, G_{var} and M_{var} served as a proxy for inter-annual heterogeneity of environmental conditions.

Study species and seed collection

From May to September 2017, we collected seeds from *Achillea millefolium*, *Bromus hordeaceus* and *Plantago lanceolata* from all plots of the Biodiversity Exploratories where the species occurred. We randomly collected ripe seeds from up to 12 individuals per plot, with a minimum distance of 1 m between those individuals. Due to ongoing management and variable seed maturity, we visited the plots several times and were able to collect seeds from 58 plots for *A. millefolium*, 69 plots for *B. hordeaceus* and 63 plots for *P. lanceolata*. We put all seeds in paper bags, dried them at room temperature and stored them at 4°C in the dark until sowing. Throughout the manuscript, we refer to seeds originating from one individual as a seed family and all individuals from one plot as a population.

Greenhouse experiment

At the end of October 2017, we sowed seeds of 5-7 seed families per population into 7 × 7 × 7 cm pots (Meyer) filled with nutrient poor potting soil (Pro Start, Geb. Brill Substrate GmbH &

Co KG, Georgsdorf). For each seed family we sowed 3 seeds each into two individual pots such that each seed family was represented in two pots. We germinated the seeds for 5 weeks in the greenhouse at 19 °C at a 12h/12h light-dark-cycle. As germination success of *P. lanceolata* was low, we additionally sowed 4 seeds from seed families that germinated poorly in the first batch into cultivation trays (PL, TK series, Pöppelmann GmbH & Co KG, Lohne) filled with the same nutrient poor soil and stratified them for 2 weeks at 4 °C in the dark (Table S1). After germination of the second batch of *P. lanceolata* plants in the greenhouse (conditions as mentioned above), we transferred two seedlings per seed family into one 7 × 7 × 7 cm pot each, filled with the same nutrient poor soil. We also individualized all seedlings from the first batch of *P. lanceolata* and all seedlings of the other two species, so that only one seedling per pot was left.

We assigned one seedling per seed family to the control group and the other one to the treatment group. We then fully randomized the plants and put each pot in an individual tray. In the second week of January 2018, we fertilized the plants of the treatment group (one plant per seed family) with an equivalent of 60 kg N ha^{-1} of a liquid NPK fertilizer (WUXAL Top N, 12-4-6 + Sp, Herman Mayer KG, Langenau). After the fertilization treatment, we grew the plants until the third week of May 2018. We watered the plants during the experiment as needed but at least two times per week. As plants got infected with mildew, white flies and aphids during the experiment, we applied hydrogen sulphide and a fungicide (TOPAS®, Syngenta Agro GmbH, Maintal**)**, and introduced biological control agents (*Encarsia formosa*, *Chrysoperla carnea*; Sautter und Stepper GmbH, Ammerbuch).

Measurements

During the second week of December 2017, we counted the leaves of all plants as a measure of initial size, especially to correct for differences between *P. lanceolata* plants from the first and second batch. Prior to the treatment, we selected and marked two representative leaves of *B. hordeaceus* and *P. lanceolata* with one silvery and one colored paper clip for measuring chlorophyll content with a SPAD-meter (SPAD 502 plus, Konica Minolta Inc., Osaka, Japan). We excluded *A. millefolium* for this measurement because of its leaf shape, which does not allow for measuring chlorophyll content with a SPAD-meter. At the day of fertilization, we took measurements of each marked leaf of *B. hordeaceus* and *P. lanceolata* while trying to exclude the midrib. We repeated this procedure 1, 3, 6, 10 and 15 days after the fertilization treatment. When a marked leaf clearly senesced or died during the course of the chlorophyll measurements, we marked and measured a third leaf which was more representative of the plants' condition. We calculated mean chlorophyll content per individual per measurement day either as the mean of the two originally marked leaves or as the mean of three leaves when we needed to include a third measurement. At day 15 after the treatment, we harvested the marked leaves of *B. hordeaceus* and *P. lanceolata* and one to three representative leaves of *A. millefolium* for leaf nitrogen and leaf carbon analysis and dried them at 70°C for 4 days. We harvested aboveground biomass, separated into reproductive and vegetative biomass, at the end of the experiment in May 2018, dried it at 70°C for four days and weighed all samples, including the leaves sampled for C:N analysis. To prepare the leaf samples for C:N analysis, we ground them in Eppendorf tubes (2 mL, Eppendorf AG, Hamburg) with 3 grinding balls (Ø 3 mm, glass, Retsch GmbH, Haan) in a mixer mill (Mixer Mill MM 400, Retsch GmbH, Haan) with 20 Hz until all material was finely ground. Carbon and nitrogen analyses were executed at the Institute of Geography and Geoecology (IFGG) at the Karlsruhe Institute of Technology (KIT). We used the average of two samples per individual as measures of mean leaf carbon and mean leaf nitrogen. We further calculated leaf C:N ratio as the quotient between mean leaf N and mean leaf C.

Statistical analysis

Sample size of the statistical analyses is smaller than that of the experiment because we only included plants that had a measurement for both control and treatment plants and populations that had data for at least 5 seed families (Table S1). To test for genetic variation in the response to the fertilization treatment, i.e. when populations differed in their response, we fitted linear mixed-effects models including number of leaves as covariate, region of origin (Exploratory), treatment, population and the interaction of the latter two as fixed effects, and seed family as random effect. We tested every species separately for vegetative biomass and total biomass (vegetative + reproductive biomass), which both included the weight of the leaf samples for C:N analysis. We applied the same linear mixed effects models to leaf N and leaf C:N ratio but used chlorophyll content at the day of the fertilization treatment as covariate for *B. hordeaceus* and *P. lanceolata* instead of number of leaves. We did not use a covariate for *A. millefolium*. To explore the pattern in the response of chlorophyll content over time in *B. hordeaceus* and *P. lanceolata*, we fitted linear mixed-effects models to the mean chlorophyll content on each day of measurement separately. We included chlorophyll content at day 0 (day of fertilization) as a covariate, region of origin (Exploratory), treatment, population and the interaction of the latter two as fixed effects, and seed family as random effect into the models.

To investigate the effects of land use on the opportunistic response to fertilization, we first calculated an index of plasticity as the log response ratio (LRR) of each previously mentioned response variable, except chlorophyll content, between fertilized and control individuals of the same seed family. To test for variation in the LRR, we fitted linear mixed-effects models including region of origin (Exploratory) and land use intensity, either F_{mean}, G_{mean} or M_{mean}, as fixed effects and population as random effect. We tested every species separately for the LRR of vegetative biomass, total biomass, leaf N and leaf C:N ratio.

To explore the effects of changes in land-use practices over time, we then fitted linear mixed-effects models including region of origin (Exploratory) and temporal variation of land-use, either F_{var}, G_{var} or M_{var}, as fixed effects and population as random effect. We again tested every species separately for the LRR of vegetative biomass, total biomass, percent N and C:N ratio.

To parameterize the strength of nitrogen acquisition in the treated plants and nitrogen conservation in the control plants, as measured by chlorophyll content, we first calculated the mean chlorophyll content for each population at day 0, which we used as a baseline for further comparisons. We then calculated the differences between chlorophyll content for every individual at every day of measurement and the baseline chlorophyll content of the respective population. As we were interested in the trade-off between nutrient acquisition and conservation, we subsequently averaged the differences for treated and untreated plants per population per measurement day and per population over all measurement days and performed Pearson´s correlation coefficient tests on these measures. To test for the association of this trade-off with land-use intensity, we additionally calculated a quotient between the measures of nutrient acquisition and conservation for each seed-family pair, averaged this per population for every measurement day and fitted linear models with region of origin and land-use intensity, either F_{mean}, G_{mean}, M_{mean}, F_{var}, G_{var} or M_{var}, as fixed effects to the quotients of every measurement day for *B. hordeaceus* and *P. lanceolata* respectively.

To meet the assumptions of homoscedasticity and normality of model residuals, we log-transformed all data for reaction norm analyses. Model residuals of all other analysis met the assumptions of homoscedasticity and normality without transformation of the response variables.

We performed all statistical analyses in R version 4.0.0 (R core team). In particular, we used the *lme4* package (Bates *et al.* 2015) and *lmerTest* package (Kuznetsova *et al.* 2017). Analyzing each species and land-use factor separately, we adjusted the false discovery rate (FDR) following Benjamini and Hochberg (1995).

Results

Population differentiation

Populations of *A. millefolium*, *B. hordeaceus* and *P. lanceolata* differed in their mean vegetative and total biomass (Table 1; Figs. 1ab, 2ab, 3ab). Additionally, populations of *A. millefolium* differed also in their response of vegetative and total biomass to the fertilization (population-by-treatment interaction, Table 1; Fig. 1ab), suggesting genetic variation among populations in their opportunistic growth response. For *B. hordeaceus*, populations only significantly differed in their response to fertilization for vegetative biomass but showed a trend for population differentiation in their growth response of total biomass (Table 1; Fig. 2ab). However, *P. lanceolata* populations did not differ in their response to fertilization, neither in vegetative nor in total biomass (Table 1; Fig 3ab), indicating a lack of genetic variation for an opportunistic growth response.

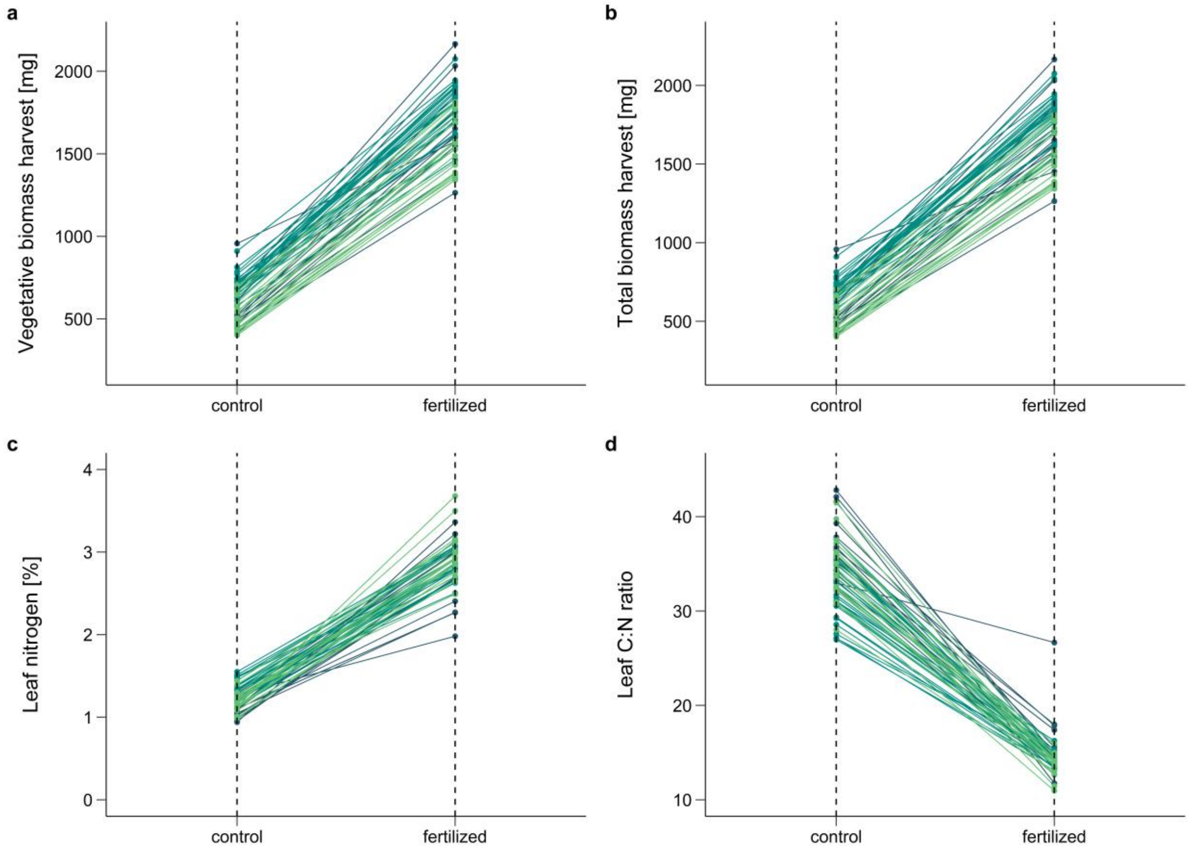


Figure 1: Reaction norm plots of *Achillea millefolium* for **a**) vegetative biomass, **b**) total biomass, **c**) percent leaf nitrogen and **d**) leaf carbon-to-nitrogen ratio (Schwäbische Alb in dark blue, Hainich in cyan, Schorfheide-Chorin in light green). Each reaction norm represents the mean values of five to seven seed families per population between the control plants and the fertilized plants.

Two weeks after fertilization, populations of all three species differed in leaf N (Table 1; Figs. 1c, 2c, 3c) and in their carbon to nitrogen ratio (Table 1; Figs. 1d, 2d, 3d). Moreover, populations of *A. millefolium* and *B. hordeaceus* also differed in the nitrogen uptake and in the response of leaf C:N ratio to fertilization (Table 1; Figs. 1c, 2cd) suggesting genetic variation among populations in the response to fertilization. Again, there was no differential response in nitrogen-related traits among *P. lanceolata* populations to fertilization (Table 1; Fig. 3cd), indicating a lack of interpopulation variation.

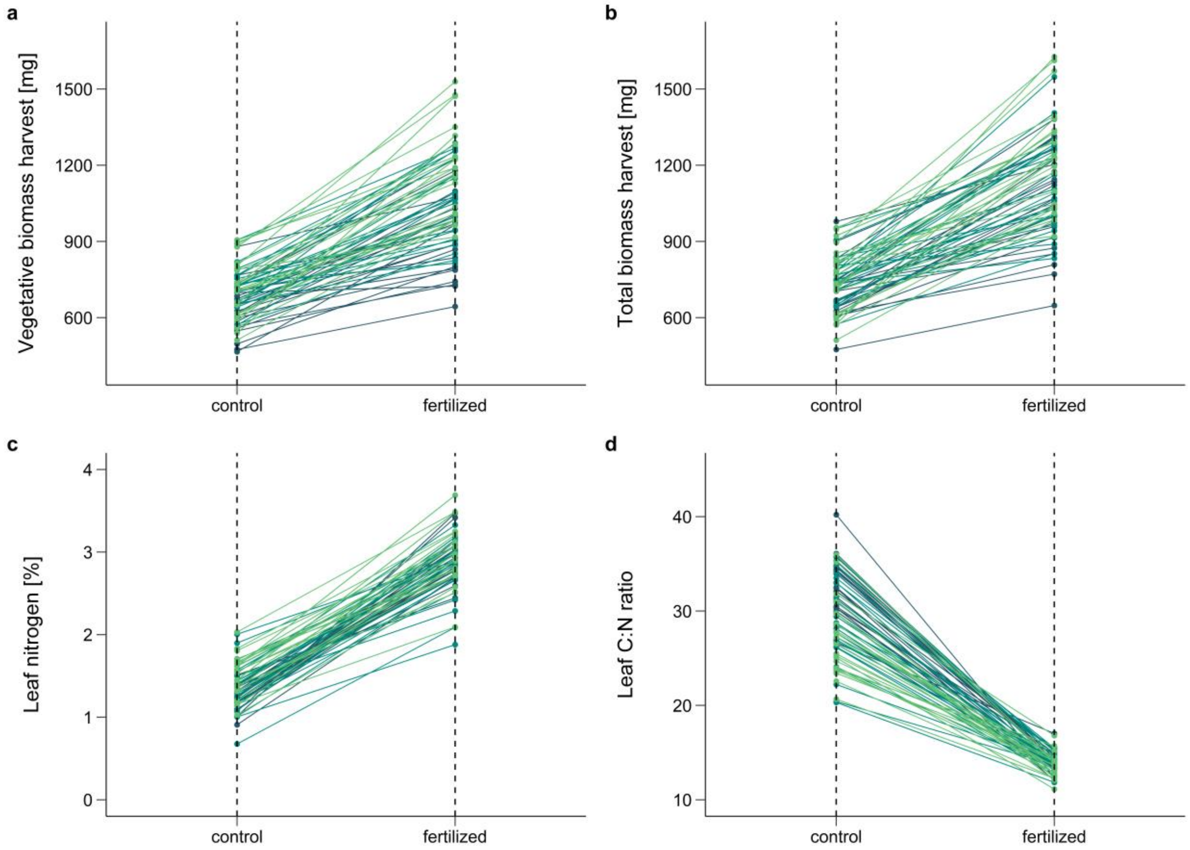


Figure 2: Reaction norm plots of *Bromus hordeaceus* for **a**) vegetative biomass, **b**) total biomass, **c**) percent leaf nitrogen and **d**) leaf carbon-to-nitrogen ratio (Schwäbische Alb in dark blue, Hainich in cyan, Schorfheide-Chorin in light green). Each reaction norm represents the mean values of five to seven seed families per population between the control plants and the fertilized plants.

Regarding chlorophyll content, the effect of fertilization was visible from day 1 after the treatment in *B. hordeaceus* but only from day 3 onwards in *P. lanceolata* (Table 2). Populations of *B. hordeaceus* only differed among each other at day 10 and day 15 after fertilization (Table 2). Furthermore, populations of *B. hordeaceus* differed in their response to fertilization at day 6 and day 10 after the treatment (Table 2). Populations of *P. lanceolata* did not differ among each other at any day of measurement (Table 2). However, variation among populations in the chlorophyll responses of *P. lanceolata* to fertilization appeared at day 10 after treatment (Table 2).

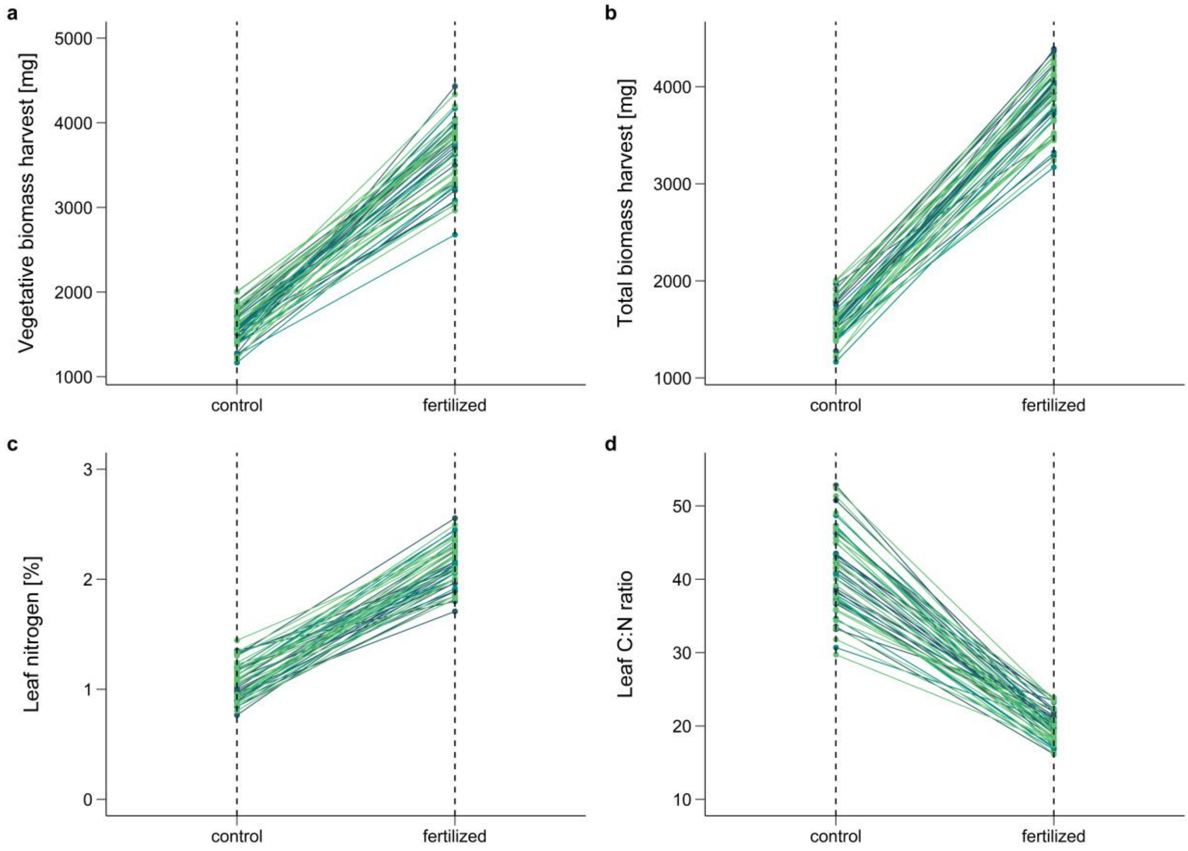


Figure 3: Reaction norm plots of *Plantago lanceolata* for **a**) vegetative biomass, **b**) total biomass, **c**) percent leaf nitrogen and **d**) leaf carbon-to-nitrogen ratio (Schwäbische Alb in dark blue, Hainich in cyan, Schorfheide-Chorin in light green). Each reaction norm represents the mean values of five to seven seed families per population between the control plants and the fertilized plants.

Table 1: Results of linear mixed-effects models of *Achillea millefolium*, *Bromus hordeaceus* and *Plantago lanceolata* testing for region of origin, population, fertilization treatment and the interaction of the latter two on vegetative biomass, total biomass, leaf nitrogen and leaf carbon-to-nitrogen ratio. Number of leaves before fertilization was used as a covariate for vegetative and total biomass and chlorophyll content before the fertilization (SPAD day 0) was used as a covariate for leaf N and leaf C:N ratio in models of *Bromus hordeaceus* and *Plantago lanceolata*. Bold *P*-values indicate significant effects ($P < 0.05$) after FDR correction. df = degrees of freedom.

		Vegetative biomass			Total biomass				Leaf N			Leaf C:N ratio		
	Num df	Den df	*F*-value	*P*-value	Den df	*F*-value	*P*-value		Den df	*F*-value	*P*-value	Den df	*F*	*P*-value
Achillea millefolium														
leaves	1	633	4.28	0.039	624	3.11	0.079		-	-	-	-	-	-
Region	2	628	5.33	**0.005**	617	5.25	**0.006**		659	3.50	**0.031**	664	1.27	0.282
Population	54	327	2.55	**<0.001**	326	2.40	**<0.001**		333	1.67	**0.004**	336	1.89	**<0.001**
Treatment	1	324	3888.65	**<0.001**	321	3889.40	**<0.001**		333	3569.26	**<0.001**	336	3589.38	**<0.001**
Pop × Treat	56	323	1.87	**<0.001**	320	1.87	**<0.001**		332	1.98	**<0.001**	335	1.96	**<0.001**
Bromus hordeaceus														
leaves	1	774	6.18	**0.013**	773	3.58	0.059	SPAD day 0	787	69.84	**<0.001**	787	51.93	**<0.001**
Region	2	738	0.41	0.665	739	0.16	0.851		772	0.47	0.627	783	1.82	0.163
Population	66	405	1.89	**<0.001**	404	2.08	**<0.001**		396	3.36	**<0.001**	399	2.12	**<0.001**
Treatment	1	397	496.99	**<0.001**	397	438.30	**<0.001**		394	2286.49	**<0.001**	399	3440.11	**<0.001**
Pop × Treat	68	395	1.43	**0.020**	394	1.29	0.071		392	1.87	**<0.001**	397	2.32	**<0.001**
Plantago lanceolata														
leaves	1	358	41.65	**<0.001**	352	59.19	**<0.001**	SPAD day 0	579	85.77	**<0.001**	579	73.89	**<0.001**
Region	2	557	1.88	0.153	554	2.02	0.133		579	0.65	0.53	579	0.72	0.49
Population	50	277	1.65	**0.007**	273	1.67	**0.006**		579	1.53	**0.013**	579	1.57	**0.010**
Treatment	1	278	1974.80	**<0.001**	275	2396.68	**<0.001**		579	1500.85	**<0.001**	579	1434.61	**<0.001**
Pop × Treat	52	277	1.01	0.463	272	1.07	0.366		579	1.27	0.103	579	1.26	0.112

Table 2: Results of linear mixed-effects models of *Bromus hordeaceus* and *Plantago lanceolata* with chlorophyll content before fertilization (SPAD day 0) as covariate for region of origin, population, fertilization treatment and the interaction of the latter two on chlorophyll content at day 1, day 3, day 6, day 10 and day 15 respectively. Bold *P*-values indicate significant effects ($P < 0.05$) after FDR correction. df = degrees of freedom.

		Chlorophyll content day 1			Chlorophyll content day 3			Chlorophyll content day 6			Chlorophyll content day 10			Chlorophyll content day 15		
	Num df	Den df	*F*	*P*	Den df	*F*	*P*	Den df	*F*	*P*	Den df	*F*	*P*	Den df	*F*	*P*
Bromus hordeaceus																
SPAD day 0	1	800	1580.51	**<0.001**	797	1766.18	**<0.001**	789	1041.19	**<0.001**	800	596.67	**<0.001**	800	206.43	**<0.001**
Region	2	800	1.94	0.144	798	1.96	0.141	800	1.01	0.364	800	0.93	0.396	800	0.49	0.614
Population	66	800	0.86	0.786	405	1.16	0.200	404	1.19	0.163	800	1.42	**0.018**	800	1.92	**<0.001**
Treatment	1	800	5.49	**0.019**	404	103.95	**<0.001**	404	1937.55	**<0.001**	800	3409.85	**<0.001**	800	2667.97	**<0.001**
Pop × Treat	68	800	1.20	0.140	404	1.27	0.088	403	1.87	**<0.001**	800	1.50	**0.008**	800	1.30	0.060
Plantago lanceolata																
SPAD day 0	1	586	3874.45	**<0.001**	587	3110.02	**<0.001**	587	2186.58	<0.001	587	1414.56	**<0.001**	587	627.17	**<0.001**
Region	2	586	0.49	0.616	587	0.020	0.980	587	0.60	0.551	587	1.22	0.296	587	0.86	0.424
Population	50	586	0.92	0.627	587	1.24	0.129	587	1.02	0.431	587	1.24	0.133	587	1.21	0.159
Treatment	1	586	2.22	0.137	587	26.40	**<0.001**	587	412.06	**<0.001**	587	1198.85	**<0.001**	587	954.19	**<0.001**
Pop × Treat	52	586	1.18	0.189	587	0.73	0.917	587	1.27	0.107	587	1.38	0.043	587	1.30	0.081

Responses to land use

Increasing mowing intensity decreased both the LRR, calculated as the log response ratio between fertilized and control plants, of vegetative and total biomass of *A. millefolium* (Table 3; Fig. 4ab). Furthermore, the LRR of leaf N two weeks after fertilization also correlated negatively with mowing intensity (Table 3; Fig. 4c). In contrast, the LRR of leaf C:N ratio correlated positively with increasing mowing intensity (Table 3; Fig. 4d). Increasing fertilization intensity decreased both the LRR of vegetative and total biomass of *A. millefolium* (Table 3; Fig. 5ab). The LRRs of leaf N and leaf C:N ratio, however, were not correlated with fertilization intensity (Table 3). All other land-use factors and the temporal variability therein did not correlate with the LRRs of all measured traits of *A. millefolium*. In *B. hordeaceus* and *P. lanceolata*, LRRs of vegetative biomass, total biomass, leaf N and leaf C:N ratio between fertilized and control plants did not correlate with any of the land-use factors or the temporal variation therein (Table 3).

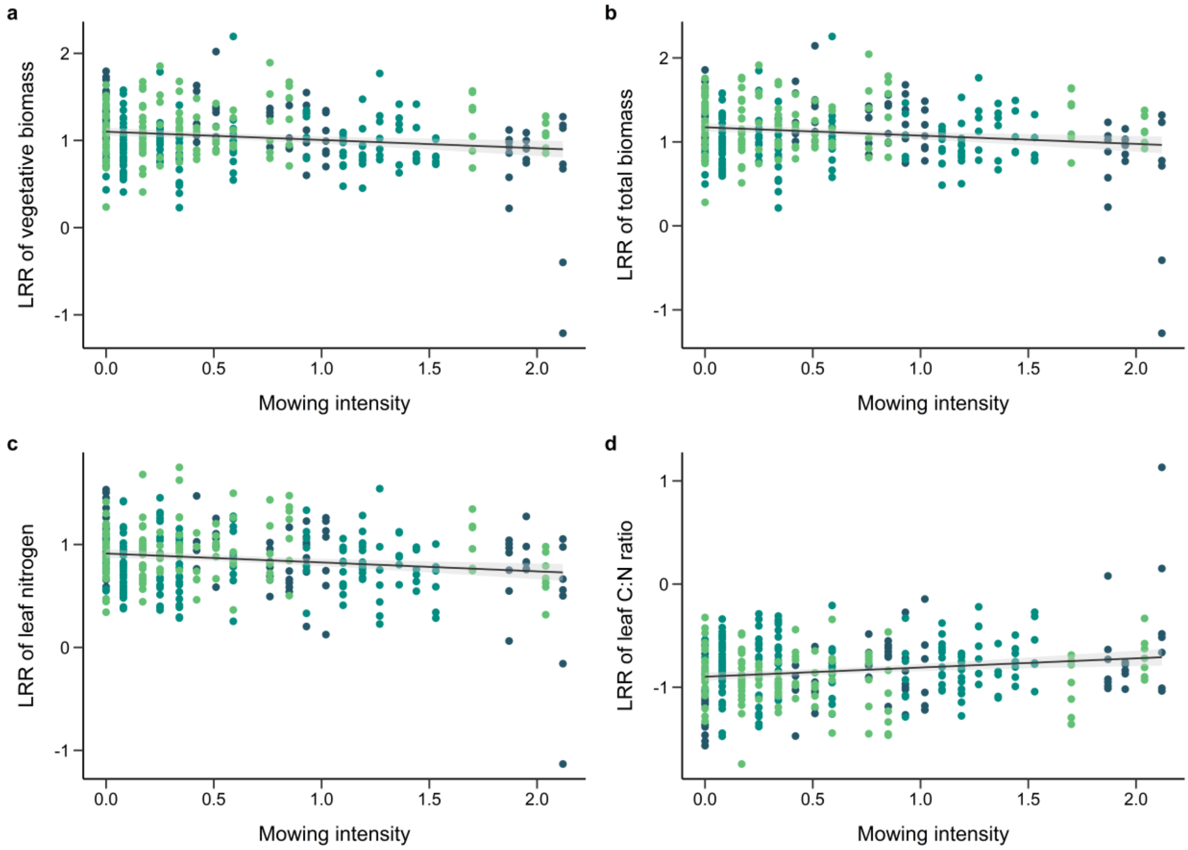


Figure 4: Relationships between mowing intensity and the plastic responses of *Achillea millefolium* to fertilization, calculated as the log response ratio (LRR) of **a**) vegetative biomass, **b**) total biomass, **c**) percent of leaf nitrogen and **d**) leaf carbon-to-nitrogen ratio (Schwäbische Alb in dark blue, Hainich in cyan, Schorfheide-Chorin in light green).

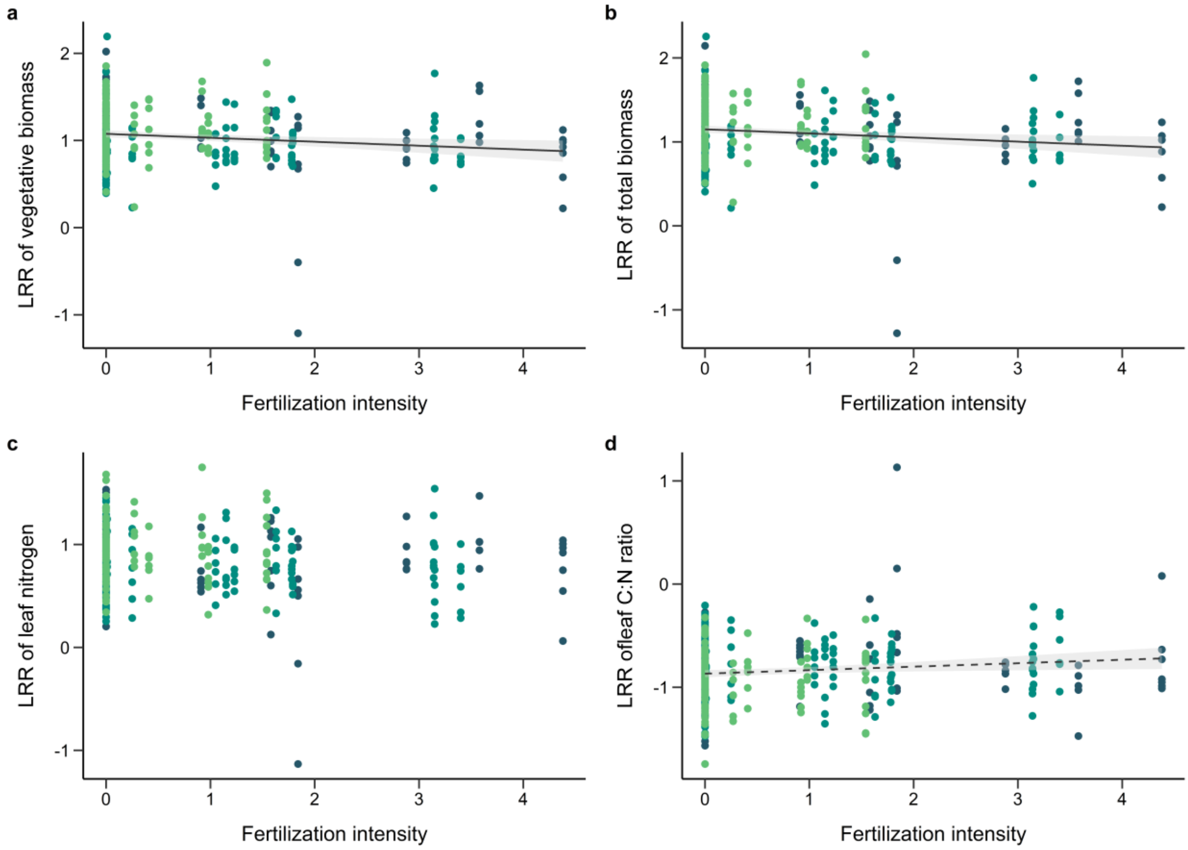


Figure 5: Relationships between fertilization intensity and the plastic responses of *Achillea millefolium* to fertilization, calculated as the log response ratio (LRR) of **a**) vegetative biomass, **b**) total biomass, **c**) percent of leaf nitrogen and **d**) leaf carbon-to-nitrogen ratio (Schwäbische Alb in dark blue, Hainich in cyan, Schorfheide-Chorin in light green).

Table 3: Results of linear mixed-effects models of *Achillea millefolium*, *Bromus hordeaceus* and *Plantago lanceolata* testing for relationships of region of origin and land use (F_{mean}, G_{mean}, M_{mean}, F_{var}, G_{var}, M_{var}) with opportunistic responses of vegetative and total biomass, leaf nitrogen and leaf carbon-to-nitrogen ratio. Population was included as a random variable. We calculated the opportunistic responses as the log response ratios (LRR) between fertilized and control individuals of the same seed-family. Land-use intensities and their temporal variations are based on land-use data from 2006-2016. We calculated temporal variation of land use as the coefficient of variation of fertilization, grazing and mowing intensities. Arrows indicate the direction of the association. Bold *P*-values indicate significant effects ($P < 0.05$) after FDR correction. df = degrees of freedom.

		LRR vegetative biomass			LRR total biomass			LRR leaf N			LRR leaf C:N ratio		
	Num df	Den df	*F*-value	*P*-value	Den df	*F*-value	*P*-value	Den df	*F*-value	*P*-value	Den df	*F*-value	*P*-value
Achillea millefolium													
Region	2	53.50	8.18	**<0.001**	51	6.79	**0.002**	53	4.53	0.015	53	4.76	0.013
F_{mean}	1	54.08	4.31	↓ 0.043	53	4.54	↓ 0.038	54	2.13	0.150	54	2.8	0.096
Region	2	54.61	6.28	**0.004**	52	5.23	0.009	54	3.21	0.048	54	3.26	0.046
G_{mean}	1	54.74	1.96	0.167	51	1.61	0.210	56	2.69	0.107	56	3.66	0.061
Region	2	53.33	9.39	**<0.001**	51	7.74	**0.001**	54	5.37	**0.008**	54	5.70	**0.006**
M_{mean}	1	50.98	8.29	↓ **0.006**	50	8.29	↓ **0.006**	53	8.15	↓ **0.006**	54	9.15	↑ **0.004**
Region	2	18.46	2.39	0.120	17.23	2.16	0.145	18.46	1.63	0.224	18.50	1.80	0.192
F_{var}	1	20.63	0.06	0.805	18.58	0.08	0.785	20.61	0.10	0.754	20.71	0.01	0.945
Region	2	320	20.41	**<0.001**	315	19.01	**<0.001**	48.34	10.21	**<0.001**	48.56	10.72	**<0.001**
G_{var}	1	320	0.35	0.558	215	0.53	0.466	46.74	0.22	0.641	46.97	0.17	0.685
Region	2	43.81	5.74	**0.006**	41.74	4.61	0.016	42.63	5.17	0.010	42.78	5.51	**0.007**
M_{var}	1	44.19	0.13	0.719	42.78	0.11	0.740	43.55	0.00	0.997	43.71	0.00	0.967
Bromus hordeaceus													
Region	2	69	3.27	0.044	69	2.59	0.083	63	4.10	0.021	62	8.29	**<0.001**
F_{mean}	1	68	0.28	0.596	68	0.03	0.856	62	0.09	0.767	61	0.38	0.538
Region	2	68	2.36	0.102	68	2.22	0.117	64	4.44	0.016	62	7.76	**<0.001**
G_{mean}	1	64	0.49	0.486	64	0.27	0.606	66	0.32	0.574	64	0.05	0.820
Region	2	69	2.72	0.073	69	2.41	0.097	63	5.33	**0.007**	62	8.28	**<0.001**
M_{mean}	1	68	0.01	0.093	68	0.04	0.844	63	0.33	0.567	61	0.33	0.568

Table 3 continued: Results of linear mixed-effects models of *Achillea millefolium*, *Bromus hordeaceus* and *Plantago lanceolata* testing for relationships of region of origin and land use (F_{mean}, G_{mean}, M_{mean}, F_{var}, G_{var}, M_{var}) with opportunistic responses of vegetative and total biomass, leaf nitrogen and leaf carbon-to-nitrogen ratio. Population was included as a random variable. We calculated the opportunistic responses as the log response ratios (LRR) between fertilized and control individuals of the same seed-family. Land-use intensities and their temporal variations are based on land-use data from 2006-2016. We calculated temporal variation of land use as the coefficient of variation of fertilization, grazing and mowing intensities. Arrows indicate the direction of the association. Bold *P*-values indicate significant effects ($P < 0.05$) after FDR correction. df = degrees of freedom.

		LRR vegetative biomass			LRR total biomass			LRR leaf N			LRR leaf C:N ratio		
	Num df	Den df	*F*-value	*P*-value	Den df	*F*-value	*P*-value	Den df	*F*-value	*P*-value	Den df	*F*-value	*P*-value
Bromus hordeaceus													
Region	2	36.93	0.04	0.960	36.93	0.05	0.957	33.82	1.73	0.193	32.92	4.26	0.023
F_{var}	1	39.16	0.06	0.816	39.40	0.10	0.748	34.37	0.94	0.340	33.32	0.15	0.699
Region	2	55.13	3.90	0.026	55.53	3.51	0.037	52.59	2.06	0.138	51.85	3.61	0.034
G_{var}	1	51.78	0.54	0.465	51.79	1.03	0.316	50.93	0.65	0.425	50.38	2.22	0.142
Region	2	57.99	1.83	0.170	57.80	1.67	0.197	53.48	6.52	**0.003**	52.07	7.03	**0.002**
M_{var}	1	59.77	0.86	0.357	59.68	1.18	0.282	56.72	1.43	0.238	54.35	0.50	0.481
Plantago lanceolata													
Region	2	47	1.45	0.244	47	2.26	0.116	48	0.74	0.482	48	0.78	0.462
F_{mean}	1	42	0.23	0.634	42	0.21	0.647	45	0.15	0.698	45	0.11	0.739
Region	2	48	1.37	0.265	48	1.77	0.172	49	1.05	0.358	49	1.17	0.321
G_{mean}	1	51	0.16	0.695	52	0.02	0.888	49	0.95	0334	48	1.01	0.320
Region	2	319	1.52	0.22	47	2.26	0.116	47	1.00	0.377	49	1.09	0.345
M_{mean}	1	319	0.64	0.43	43	0.27	0.605	46	0.79	0.379	46	0.80	0.375
Region	2	21.09	0.30	0.746	20.96	0.92	0.416	22.36	0.34	0.714	22.18	0.29	0.751
F_{var}	1	22.04	0.05	0.834	21.71	0.34	0.568	20.56	2.03	0.169	20.46	1.71	0.206
Region	2	245	0.72	0.488	241	1.56	0.212	254	0.69	0.505	254	0.79	0.457
G_{var}	1	245	0.00	0.955	241	0.02	0.896	254	0.00	0.980	254	0.00	0.966
Region	2	227	1.13	0.324	40.96	1.76	0.184	42.22	1.08	0.350	42.11	1.04	0.363
M_{var}	1	277	1.06	0.304	39.15	0.60	0.442	39.79	1.01	0.322	39.71	0.83	0.367

Acquisition vs. conservation trade-off

Measures of overall nutrient acquisition and conservation correlated negatively for both *B. hordeaceus* and *P. lanceolata* (Table 4; Fig. 6). For specific measurement days, the negative associations were very strong (Table 4; Fig. 6). However, for *P. lanceolata* the strength of the correlation clearly decreased at day 15 and for *B. hordeaceus* the correlation even turned positive at day 15 (Table 4; Fig. 6).

Table 4: Results of *Bromus hordeaceus* and *Plantago lanceolata* of Pearson´s correlation coefficient tests between the mean differences of chlorophyll content of fertilized and control individuals to population mean chlorophyll content before fertilization. Test statistics are given for chlorophyll content measurements on day 1, day 3, day 6, day 10, and day 15 after fertilization as well as for a global correlation across all measurements.

	Bromus hordeaceus		*Plantago lanceolata*	
	r	*P*	r	*P*
Day 1	- 0.55	< 0.001	- 0.76	< 0.001
Day 3	- 0.41	< 0.001	- 0.63	< 0.001
Day 6	- 0.35	0.003	- 0.54	< 0.001
Day 10	- 0.16	0.200	- 0.45	< 0.001
Day 15	0.23	0.056	- 0.29	0.036
Overall	- 0.56	< 0.001	- 0.25	< 0.001

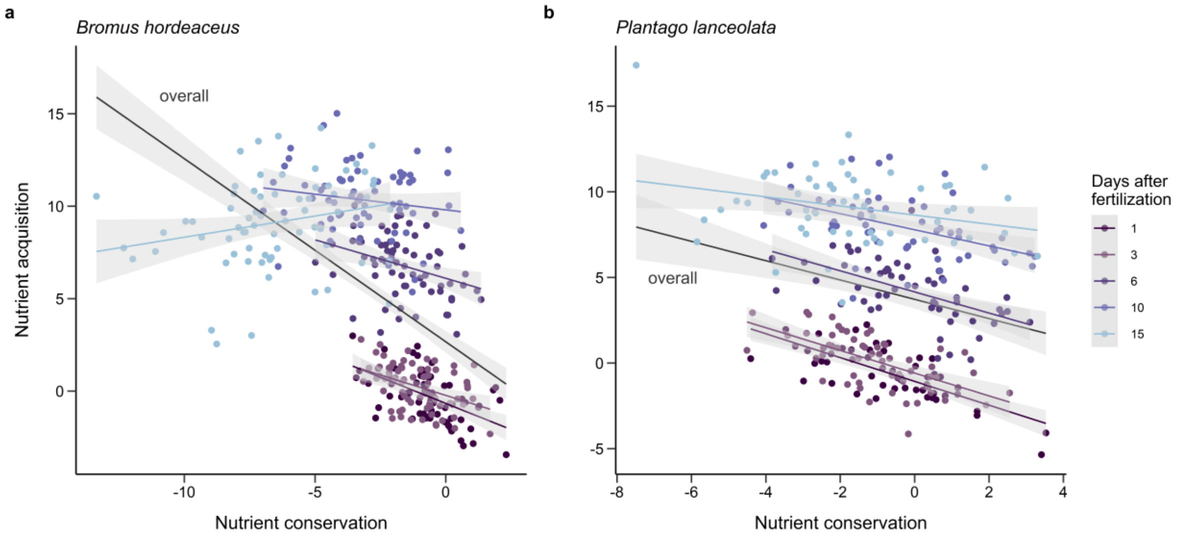


Figure 6: Relationship between nutrient conservation and acquisition in **a**) *Bromus hordeaceus* and **b**) *Plantago lanceolata*, calculated as the differences between mean chlorophyll content of fertilized plants respectively control plants and mean chlorophyll content before the fertilization for each day of measurement after fertilization and across all measurements (grey line).

Nutrient acquisition i.e. incorporation of nitrogen into leaf chlorophyll, started shortly after the fertilization and increased more or less strongly over the course of two weeks steadily in both species (Fig. 7, S1, S2). Levels of nutrient conservation i.e. retention of leaf N measured as leaf chlorophyll content, of *P. lanceolata* populations were rather low and constant over time

(Fig. 7cd, S2) whereas leaf N retention of *B. hordeaceus* populations was lower compared to *P. lanceolata* and decreased over time (Fig. 7ab, S1). The strength of the acquisition conservation trade-off, calculated as the quotient between nutrient acquisition and nutrient conservation, was negatively associated with temporal variation in fertilization intensity for *B. hordeaceus* at day 15 and with temporal variation in grazing intensity for *P. lanceolata* at day 3 (Table S2). However, the negative correlation of the acquisition conservation trade-off with temporal variation in *B. hordeaceus* is most likely triggered by one extreme quotient (Fig. S3).

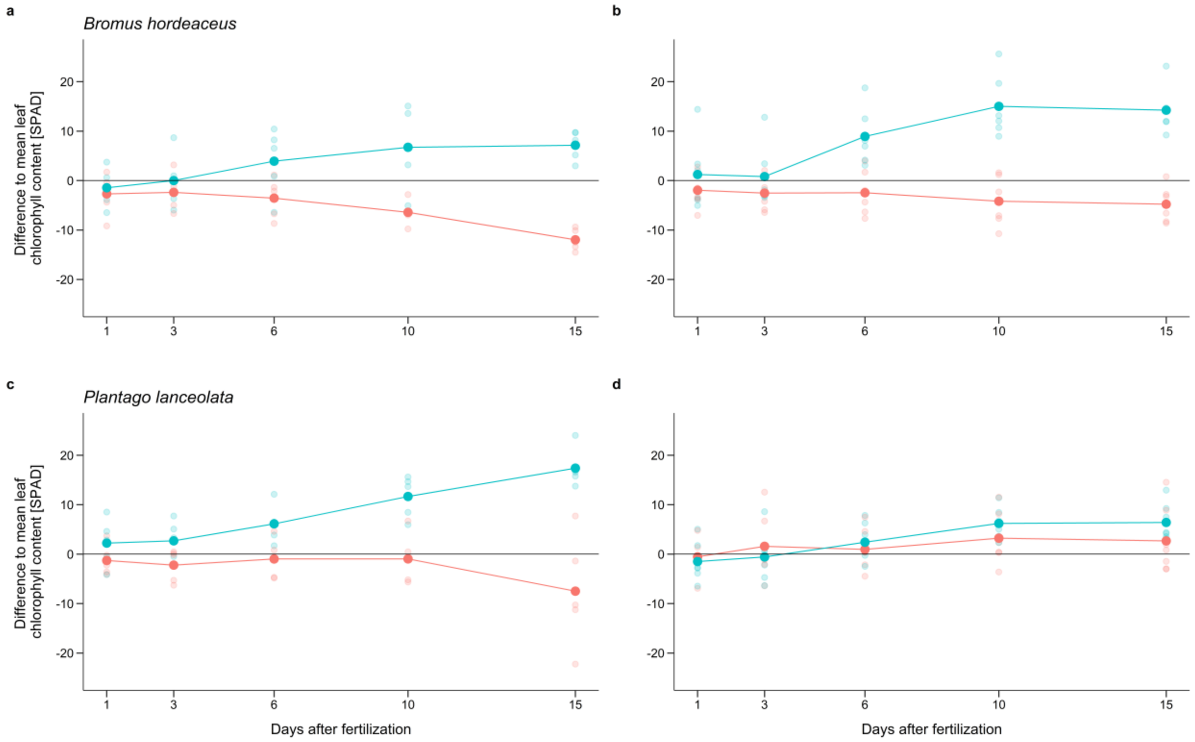


Figure 7: Exemplary trends of chlorophyll content in fertilized plants (blue) and in control plants (red) of **a**) and **b**) *Bromus hordeaceus* and **c**) and **d**) of *Plantago lanceolata* over the course of two weeks after the fertilization treatment, calculated as the difference between population mean (5-7 seed families) chlorophyll content at day 0 (before the treatment) and population mean chlorophyll content at day 1, day 3, day 6, day 10 and day 15 after the fertilization treatment, respectively. Larger dark-hued points represent means of fertilized and control individuals, whereas smaller light-hued points represent single individuals. Panels **a**) and **b**) present two example populations of *Bromus hordeaceus*, and panels **c**) and **d**) two example populations of *Plantago lanceolata*. Panels **b**) and **c**) show a strong increase of chlorophyll content after fertilization (acquisition), whereas the increase in chlorophyll content in panels **a**) and **d**) are less pronounced. In panels **a**) and **c**) the chlorophyll content decreases (conservation) over time, whereas chlorophyll content stays rather constant in panels **b**) and **d**).

Discussion

Type and intensity of land use, one of the biggest global change drivers, often differ strongly among grasslands and thus the resident plant populations are expected to locally adapt. The evolutionary consequences of land use on trait means has been studied extensively, however studies on the evolution of plasticity remain scarce. In this common garden study, we investigated if plants from a large number of grasslands along a gradient of land-use intensity evolved locally adapted opportunistic responses to fertilization in biomass and leaf nitrogen-related traits. We found significant population differentiation in the way that *Achillea millefolium* plants responded to fertilization in all traits examined. The strength of these plastic responses correlated negatively with mowing and fertilization intensity of the grasslands of origin. Contrastingly, genetically-based trait variation of populations in *Bromus hordeaceus* did not associate with land-use intensity. Additionally, populations of *Bromus hordeaceus* and *Plantago lanceolata* exhibited differences in their response of leaf chlorophyll content to fertilization on a short term, which ceased again two weeks after fertilization. These population differences were also apparent in a trade-off between acquisition and conservation of leaf chlorophyll content such that populations that were better at increasing their leaf chlorophyll after fertilization were less good in conserving leaf chlorophyll under limited nutrient supply and vice versa.

Population differentiation

The discrepancy in our results regarding population differentiation among our three study species could be discussed in light of their breeding system. In *A. millefolium*, evolution of locally different responses towards fertilization seems plausible, as this species is insect-pollinated and has rather short seed dispersal distances (Bourdôt and Field 1988), which reduces gene flow among populations and facilitates population differentiation. Similarly, in *B. hordeaceus*, a largely selfing species, the evolution of different responses among populations might be inevitable. This is also supported by variation in trait means such as onset of flowering or plant height among *B. hordeaceus* plants from an overlapping set of populations (Völler *et al.* 2013, 2017). Contrastingly, gene flow among populations of *P. lanceolata* is expected to be high as this species is a wind-pollinated obligate outcrosser, which could prevent population differentiation as seen among our study populations. Similarly, no differentiation among populations in this system has been found in the nutrient pulse response measured as leaf chlorophyll content two weeks after fertilization (Gáspár *et al.* 2020). However, we measured leaf chlorophyll content on several dates within two weeks after fertilization and we found that *P. lanceolata* populations, but also *B. hordeaceus* populations, differed in their chlorophyll response 10 and 6 days after the nutrient pulse, respectively, but this differentiation ceased again after two weeks. This indicates differences in short-term nutrient responses among populations and highlights the importance of continuous measurements (cf. "process trait",Volaire *et al.* 2020), as we could have missed this differentiation if we took a measurement at a single moment only.

Another possible explanation for a lack of population differentiation in the response to fertilization in *P. lanceolata* aboveground traits could be this species' strategy to store resources in the form of non-structural carbohydrates in its taproot (Latzel, Janeček, Hájek, *et al.* 2014). In a perennial species, storage of nutrients might serve as a strategy to buffer against times of nutrient limitation and might thus be under selection. Classic as well as recent literature shows that plants often display morphological and physiological plasticity in root traits, such as root length, root diameter or uptake capacity, in response to nutrients (Boot and Mensink 1990;

Hutchings and de Kroon 1994; Fransen *et al.* 1998; Wijesinghe *et al.* 2001; Hodge 2004). Thus, it is very likely that *P. lanceolata* also responds in belowground traits to fertilization, especially in terms of nutrient storage in its taproot, and probably evolved population differentiation in respect to this nutrient storage function. In contrast, *B. hordeaceus* is an annual species with a fast life cycle that might as well show belowground foraging plasticity in response to nutrients. However, as its nutrient storage capacity is limited (cf *P. lanceolata*) it might just invest them into aboveground growth, and possible population differentiation might then manifest in aboveground biomass or nitrogen related traits. The discussed species differences show that each has its specific life history and functional traits that cause it to respond and evolve differently.

Relationships of trait plasticity with land use – inter-and intra-annual variation

Our next question was whether any population differentiation in trait responses to fertilization correlates with local environmental conditions, in our study focusing on land-use practices. Heterogeneous environmental conditions, such as created by common land-use practices, are thought to select for plasticity (Sultan 1987; Alpert and Simms 2002; Scheiner 2013). However, to our knowledge only one other study investigated the direct association of land-use intensity, a proxy for heterogeneous environmental conditions, and the magnitude of plant responses i.e. plasticity, to nutrient supply (Gáspár *et al.* 2020). There was no evidence that biomass and nitrogen-related responses of *B. hordeaceus* and *P. lanceolata* are associated with land-use type and intensity in our study. As *B. hordeaceus* exhibited population differentiation in the responses to fertilization in three out of four traits and associations of trait means with land use are present in this species (Völler *et al.* 2013, 2017), we expected that differences in plasticity would also have evolved in relation to land use. However, population differentiation, especially in species with low gene flow such as *B. hordeaceus*, can also emerge as results of genetic drift or genetic linkage (Hartfield *et al.* 2017).

In contrast, we found relationships between the strength of plasticity and land use in *A. millefolium*, where trait responses associated most strongly with mowing intensity and to a lesser extent with fertilization intensity. It is intuitive that both management practices selected for the same pattern of differentially expressed plasticity, as they correlate positively in our study system (Blüthgen *et al.* 2012; Völler *et al.* 2017). Contrastingly, plasticity in leaf C:N ratio correlated positively with land-use intensity. Since the log response ratio (LRR) of leaf C:N ratio is negative, fertilized plants had a lower C:N ratio than control plants. This contrasts with the positive LRRs of the other traits, generally reflecting higher trait values of fertilized plants than control plants while decreasing with increasing land use intensity. When we assume a rather stable C fraction in both fertilized and control plants (Fig. S4, S5 and S6) this indicates that fertilized plants had higher leaf N than control plants. This positive correlation of the LRR of C:N ratio with land use intensity therefore also suggests a decreased N uptake in more frequently mown and fertilized grasslands, as seen in leaf chlorophyll content. As we, on the one hand, hypothesized that increasing management represents increasing environmental heterogeneity (heterogenization hypothesis), these negative associations challenge the common view that plasticity should be advantageous and evolve in heterogeneous environments (Scheiner 2013). However, these negative associations instead support our homogenization hypothesis, that plasticity should be weaker under more intensive land use. One reason for this result could be that intensively managed plots along the land-use gradient receive high amounts of fertilizer at several occasions a year. This probably rather homogenizes nutrient conditions over the course of the year (Leiss and Müller-Schärer 2001), whereas the extensively and intermediately intensively managed plots may in fact represent a more fluctuating environment in terms of nutrient availability.

Besides the common view that plasticity should evolve in heterogeneous environments, classical theoretical and empirical studies (Davies and Snaydon 1974; Chapin 1980; Aerts and Chapin 1999) specifically hypothesized that plasticity should be stronger in high fertility soils compared to low fertility soils, which is represented by high vs. low fertilization intensity in our study, respectively. However, our finding that plasticity of *A. millefolium* is lower in more intensively fertilized plots i.e. plots with higher soil fertility, questions this general opinion. Yet, in contrast to this classical literature, some more recent studies likewise found that plants from less fertile habitats showed higher plasticity than their counterparts from habitats that are more fertile did (Osone and Tateno 2005; Vergeer *et al.* 2008; Fritz *et al.* 2014; Wedlich *et al.* 2016). Wedlich and colleagues (2016) for example, found that *Prunella vulgaris* plants from low N accessions exhibited a higher N use efficiency than plants from medium or high N accessions. This is in accordance with an earlier study of Vergeer and colleagues (2008) on atmospheric nitrogen deposition rates, that also reported higher N use efficiency plasticity in *Arabidopsis lyrata petraea* plants from low N deposition regions. In contrast, *Senecio vulgaris* plants from an agricultural habitat characterized by high fertilizer input showed higher plasticity in response to nutrient addition in reproductive biomass and leaf area than plants from a ruderal site with low nutrient availability (Leiss and Müller-Schärer 2001). Given these mixed results and the potential of high fertilization intensity to homogenize growing conditions, it might well be possible that *A. millefolium* evolved higher plasticity under low fertilization intensity.

Since farmers vary their management practices in terms of type and intensity (e.g. stocking densities, mowing frequency, amount of fertilizer) from year to year, we hypothesized that the greater these changes are, i.e. higher temporal variation in management, the stronger the plant responses to nutrient supply should be. However, we did not find any patterns of plasticity with temporal variation in land use. Nevertheless, in a different study on patterns of plasticity in response to clipping, we found that regrowth ability of reproductive biomass in *P. lanceolata* was higher under a more constant mowing regime (Kirschbaum *et al.* 2021), highlighting the importance of predictability of environmental conditions for the evolution of plasticity (Scheiner 1993; Stuefer 1996; Alpert and Simms 2002; Lande 2009; Reed *et al.* 2010). The discrepancy between the results of these two studies might be explained by the characteristics of the two management types. Mowing on the one hand is a discrete event whereas fertilization on the other hand is a discrete event as well but has the potential to change soil nutrient conditions over a longer period of time. As such fertilization might not be as predictable anyways, preventing the evolution of plasticity to inter-annual temporal variation.

Acquisition conservation trade-off

We found a clear trade-off in terms of leaf chlorophyll increase after fertilization and decrease in control plants in both *B. hordeaceus* and *P. lanceolata*. Populations that were better in increasing leaf chlorophyll content after a fertilization event were worse in retaining chlorophyll content under limited conditions and vice versa. We believe that the trade-off leveling off after two weeks is a sign for decreasing nutrient uptake rate, either because most nutrients have been taken up by that time or because the plants just cannot incorporate more nitrogen because of imminent intoxification (Fritz *et al.* 2014). As the marked leaves for chlorophyll content measurements at day 10 and day 15 often showed distinct symptoms of leaf senescence (e.g. yellowish color), we often took a third measurement on a leaf that was more representative of the plants' condition. Hence, the decrease in chlorophyll content is not only the result of senescence processes but reflects the ability of a plant to conserve leaf nitrogen. This shows that population differences in traits related to plant nutrient dynamics can be transient and can best be investigated over time, i.e. as a "process trait" rather than a "pattern trait" (Volaire *et al.* 2020).

The trade-off between nutrient acquisition (response after fertilization) and conservation (response under limited nutrient conditions) reflects a well-known specialization of contrasting life-history strategies along an acquisition-conservation axis (Díaz *et al.* 2004, 2016; Reich 2014). These strategies are well reflected by a suite of morphological, physiological and chemical leaf traits (leaf economics spectrum - LES (Wright *et al.* 2004; Shipley *et al.* 2006; Reich 2014)) and to a lesser extent by root traits (root economics spectrum - RES (Reich 2014; Roumet *et al.* 2016; Weemstra *et al.* 2016)), that often form specific functional trait syndromes. These trait syndromes are well-characterized across species, but in recent years the importance of intraspecific trait variation and the potential for within-species trade-offs has been acknowledged (Niinemets 2015; Gagliardi *et al.* 2015; Isaac *et al.* 2017; Martin *et al.* 2017; Sartori *et al.* 2019). Instead of exploring bi- or multivariate trait correlations for trade-offs, we employed the strategy to study the responses of increasing vs. decreasing nutrient availability in a single trait and hence capture real response strategies. Trait syndrome variation is often structured along environmental gradients such as climate or land use. Studies that likewise found marked intraspecific plant strategy trade-offs could relate this differentiation to climate (Sartori *et al.* 2019), light transmittance (Gagliardi *et al.* 2015) and agricultural management (Martin *et al.* 2017), showing that several biotic, but probably also abiotic factors could shape such trade-offs. However, in our study the observed trade-off did not consistently associate with land use. As mentioned earlier, the negative association of the acquisition conservation trade-off at day 15 after fertilization and temporal variation in fertilization intensity for *B. hordeaceus* is most likely triggered by one extreme quotient and thus not trustworthy. However, the negative association between the acquisition conservation trade-off at day 3 after fertilization and temporal variation in grazing intensity for *P. lanceolata* is more reliable, although the lack of correlations at day 1 and day 6 make this a rather conspicuous result. Nevertheless, as the effect of fertilization was first detectable at day 3 after the treatment in *P. lanceolata*, this association could be representative of the time of most nutrient uptake. However, how this association would constitute an adaptive advantage is difficult to conceive. Additionally, in our study system along a north-south transect in Germany, land use might not be the only environmental gradient present, but factors such as climate or other soil parameters might also represent environmental gradients that could have shaped the observed trade-off in chlorophyll acquisition and conservation.

In our study, the implementation of many populations along a gradient of land-use intensity, allowed us the detection of clear patterns in phenotypic plasticity. However, clear genetic variation in plasticity could not adequately be explained by the environmental variables we employed. Therefore, we propose to consider other potential drivers on the evolution of plasticity including more environmental variables.

Funding

The work was supported by the DFG Priority Program 1374 "Infrastructure-Biodiversity-Exploratories" through project SCHE 1899/1-1 to JFS.

Acknowledgments

We thank the managers of the three Exploratories, Kirsten Reichel-Jung, Iris Steitz, Sandra Weithmann, Juliane Vogt and Miriam Teuscher, and all former managers for their work in maintaining the plot and project infrastructure,Christiane Fischer for giving support through the central office, Andreas Ostrowski for managing the central data base, and Markus Fischer, Eduard Linsenmair, Dominik Hessenmöller, Daniel Prati, Ingo Schöning, François Buscot, Ernst-Detlef Schulze, Wolfgang W. Weisser and the late Elisabeth Kalko for their role in setting up the Biodiversity Exploratories project. The work has been funded by the DFG Priority Program 1374 "Infrastructure-Biodiversity-Exploratories" through project SCHE 1899/1-1 to JFS. Field work permits were issued by the responsible state environmental offices of Baden-Württemberg, Thüringen, and Brandenburg. We also thank Andre Velescu (KIT) for the leaf C and N analysis and Christiane Karasch-Wittmann, Leah Kirchhoff, Eva Schloter, Tabea Schwarz, Melissa Sehrt, Sabine Silberhorn, Lea Stier and Simone Vogel for help with seed sampling and experimental measurements.

Supplements

Table S1: Key life-history traits of our three study species and numbers of individuals and populations that we used in the experiment and in the statistical analyses. Additionally, the numbers of individuals per batch of *Plantago lanceolata* are given, as we resowed several seed families (2. batch).

	Achillea millefolium	*Bromus hordeaceus*	*Plantago lanceolata*
Plant family	Asteraceae	Poaceae	Plantaginaceae
Pollination type	Insects	Self/Wind	Wind
Breeding system	Obligate outcrosser	Predominantly autogamous	Obligate outcrosser
Life cycle	Perennial	Annual	Perennial
# Individuals (experiment/analyses)	790/780	960/942	712/696
1. batch	-/-	-/-	488/476
2. batch	-/-	-/-	224/220
# Populations (experiment/analyses)	58/57	69/69	55/53

Table S2: Results of linear models for *Bromus hordeaceus* and *Plantago lanceolata* testing the relationships of region of origin and land use (F_{mean}, G_{mean}, M_{mean}, F_{var}, G_{var}, M_{var}) with the acquisition conservation trade-off, calculated as a quotient between the measures of nutrient acquisition and conservation for each population for every day chlorophyll content was measured at (day1, day 3, day 6, day 10 and day 15 after fertilization). Land-use intensities and their temporal variations are based on land-use data from 2006-2016. We calculated temporal variation of land use as the square root of the standard deviations of fertilization, grazing and mowing intensities and the combined intensities of grazing and mowing, and, fertilization and grazing, respectively. Bold *P*-values indicate significant effects ($P < 0.05$) after FDR correction. df = degrees of freedom.

		Day 1		Day 3		Day 6		Day 10		Day 15	
	df	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Bromus hordeaceus											
Region	2	0.91	0.409	1.13	0.331	2.51	0.090	1.66	0.198	1.05	0.358
F_{mean}	1	0.99	0.323	0.05	0.822	0.17	0.680	1.15	0.288	0.41	0.524
Region	2	0.90	0.412	1.13	0.330	2.54	0.086	1.68	0.194	1.07	0.350
G_{mean}	1	0.51	0.476	0.09	0.761	1.17	0.283	1.90	0.173	1.73	0.193
Region	2	0.91	0.406	1.13	0.331	2.51	0.089	1.64	0.201	1.04	0.359
M_{mean}	1	1.56	0.216	0.03	0.858	0.38	0.540	0.37	0.546	0.26	0.613
Region	2	0.73	0.489	0.19	0.832	1.27	0.292	3.07	0.059	0.79	0.464
F_{var}	1	0.04	0.845	1.43	0.239	0.03	0.868	3.39	0.074	8.45	↓ 0.006
Region	2	0.67	0.517	0.80	0.455	2.39	0.102	0.73	0.485	0.93	0.400
G_{var}	1	0.02	0.902	0.07	0.797	1.03	0.314	0.10	0.752	1.35	0.251
Region	2	0.75	0.476	0.19	0.824	2.17	0.124	1.68	0.197	1.01	0.371
M_{var}	1	1.74	0.193	0.16	0.695	1.01	0.320	1.09	0.302	0.11	0.741

Table S2 continued: Results of linear models for *Bromus hordeaceus* and *Plantago lanceolata* testing the relationships of region of origin and land use (F_{mean}, G_{mean}, M_{mean}, F_{var}, G_{var}, M_{var}) with the acquisition conservation trade-off, calculated as a quotient between the measures of nutrient acquisition and conservation for each population for every day chlorophyll content was measured at (day1, day 3, day 6, day 10 and day 15 after fertilization). Land-use intensities and their temporal variations are based on land-use data from 2006-2016. We calculated temporal variation of land use as the square root of the standard deviations of fertilization, grazing and mowing intensities and the combined intensities of grazing and mowing, and, fertilization and grazing, respectively. Bold *P*-values indicate significant effects ($P < 0.05$) after FDR correction. df = degrees of freedom.

		Day 1		Day 3		Day 6		Day 10		Day 15	
	df	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Plantago lanceolata											
Region	2	0.74	0.481	0.31	0.735	0.37	0.693	1.16	0.322	0.72	0.493
F_{mean}	1	0.05	0.827	0.29	0.592	0.08	0.775	3.44	0.070	0.13	0.723
Region	2	0.80	0.456	0.31	0.736	0.37	0.693	1.09	0.344	0.74	0.484
G_{mean}	1	3.56	0.065	0.15	0.703	0.00	0.991	0.16	0.695	1.46	0.233
Region	2	0.74	0.480	0.31	0.735	0.37	0.692	1.09	0.344	0.72	0.492
M_{mean}	1	0.08	0.777	0.28	0.597	0.18	0.670	0.27	0.606	0.27	0.605
Region	2	1.01	0.381	0.04	0.960	2.73	0.086	0.81	0.457	2.49	0.105
F_{var}	1	2.37	0.138	1.15	0.295	0.00	0.978	0.01	0.916	0.23	0.633
Region	2	0.41	0.669	0.77	0.472	0.41	0.665	1.54	0.228	0.65	0.526
G_{var}	1	0.02	0.901	5.91	↓ 0.020	0.00	0.954	1.60	0.213	3.79	0.059
Region	2	1.09	0.345	0.15	0.864	1.95	0.155	1.10	0.343	0.49	0.618
M_{var}	1	0.10	0.756	0.10	0.751	0.00	0.960	0.00	0.957	1.34	0.253

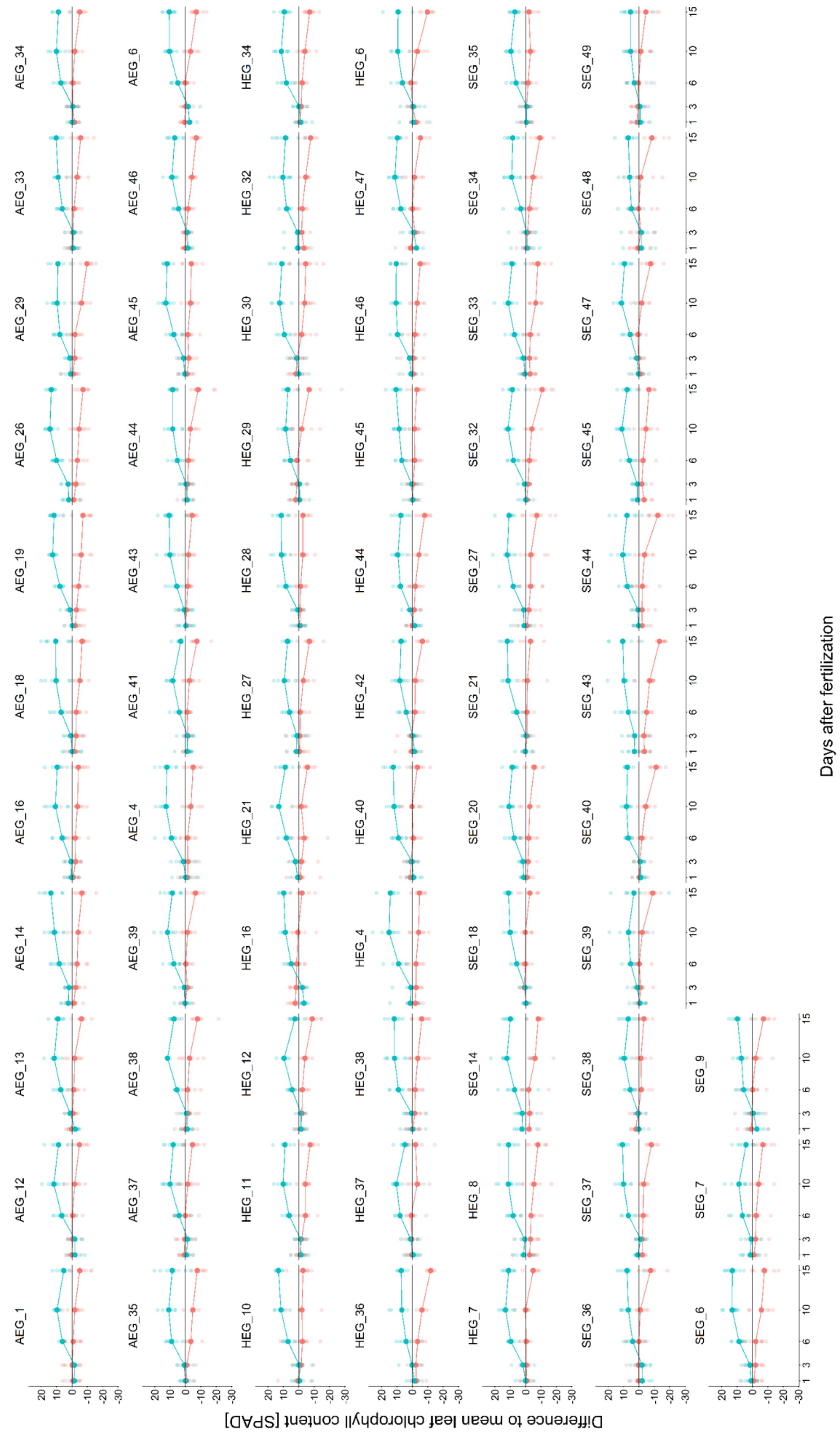

AEG_1
AEG_12
AEG_13
AEG_14
AEG_16
AEG_18
AEG_19
AEG_26
AEG_29
AEG_33
AEG_34
AEG_35
AEG_37
AEG_38
AEG_39
AEG_4
AEG_41
AEG_43
AEG_44
AEG_45
AEG_46
AEG_6
HEG_10
HEG_11
HEG_12
HEG_16
HEG_21
HEG_27
HEG_28
HEG_29
HEG_30
HEG_32
HEG_34
HEG_36
HEG_37
HEG_38
HEG_4
HEG_40
HEG_42
HEG_44
HEG_45
HEG_46
HEG_47
HEG_6
HEG_7
HEG_8
SEG_14
SEG_18
SEG_20
SEG_21
SEG_27
SEG_32
SEG_33
SEG_34
SEG_35
SEG_36
SEG_37
SEG_38
SEG_39
SEG_40
SEG_43
SEG_44
SEG_45
SEG_47
SEG_48
SEG_49
SEG_6
SEG_7
SEG_9
20
10
0
-10
-20
-30
1
3
6
10
15
Difference to mean leaf chlorophyll content [SPAD]
Days after fertilization

Figure S1: Trends of chlorophyll acquisition and conservation in *Bromus hordeaceus* from fertilized plants (blue) and control plants (red) over the course of two weeks after the fertilization treatment. We calculated acquisition and conservation per population as the (mean) difference of fertilized and control individuals at day 1, day 3, day6, day 10 and day 15 to mean population chlorophyll content at the day of fertilization. Larger dark-hued points represent means of fertilized and control individuals, whereas smaller light-hued points represent single individuals.

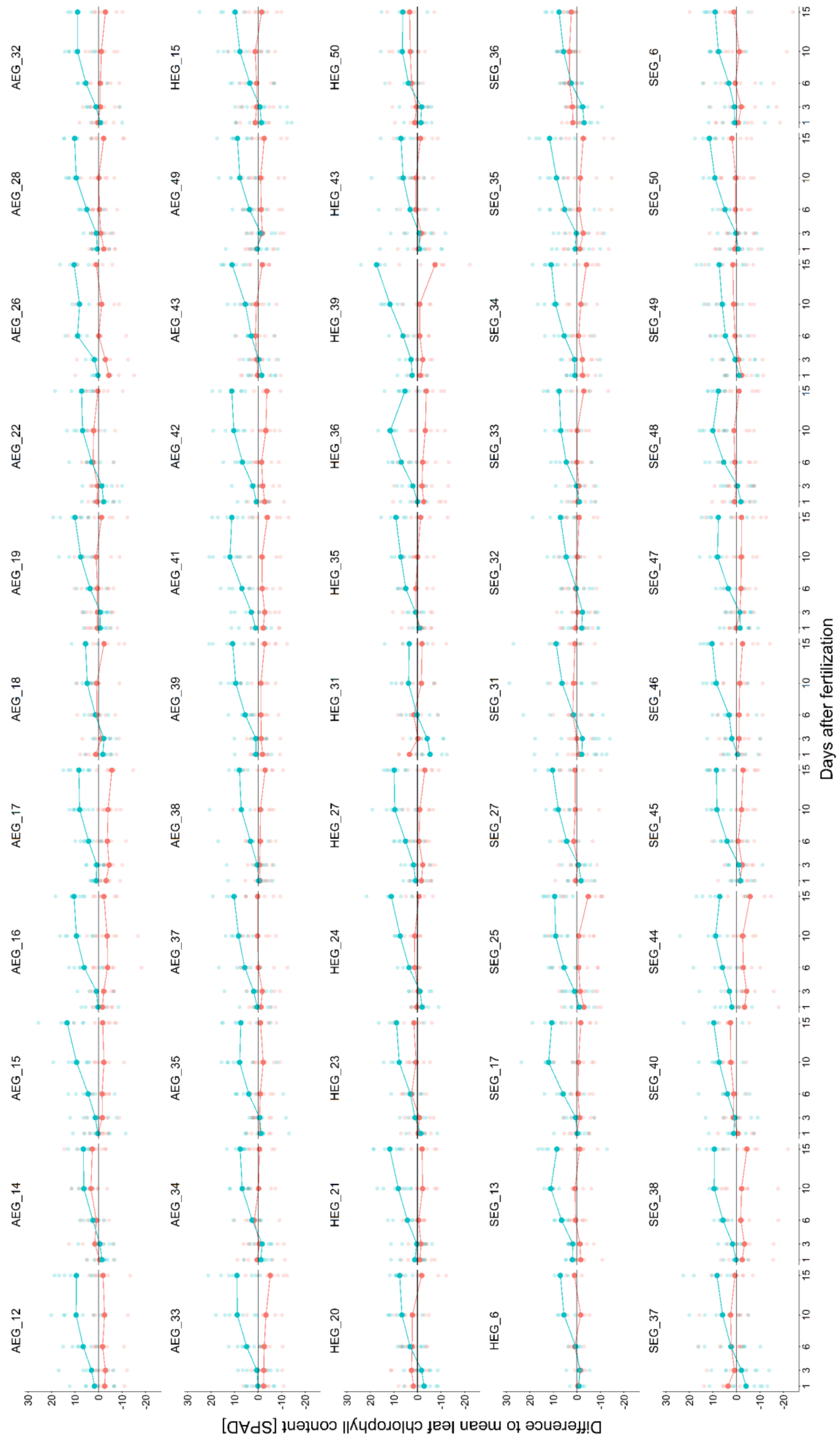

AEG_12
AEG_14
AEG_15
AEG_16
AEG_17
AEG_18
AEG_19
AEG_22
AEG_26
AEG_28
AEG_32
AEG_33
AEG_34
AEG_35
AEG_37
AEG_38
AEG_39
AEG_41
AEG_42
AEG_43
AEG_49
HEG_15
HEG_20
HEG_21
HEG_23
HEG_24
HEG_27
HEG_31
HEG_35
HEG_36
HEG_39
HEG_43
HEG_50
HEG_6
SEG_13
SEG_17
SEG_25
SEG_27
SEG_31
SEG_32
SEG_33
SEG_34
SEG_35
SEG_36
SEG_37
SEG_38
SEG_40
SEG_44
SEG_45
SEG_46
SEG_47
SEG_48
SEG_49
SEG_50
SEG_6
Difference to mean leaf chlorophyll content [SPAD]
30
20
10
0
-10
-20
Days after fertilization
1
3
6
10
15

Figure S2: Trends of chlorophyll acquisition and conservation in *Plantago lanceolata* from fertilized plants (blue) and control plants (red) over the course of two weeks after the fertilization treatment. We calculated acquisition and conservation per population as the (mean) difference of fertilized and control individuals at day 1, day 3, day6, day 10 and day 15 to mean population chlorophyll content at the day of fertilization. Larger dark-hued points represent means of fertilized and control individuals, whereas smaller light-hued points represent single individuals.

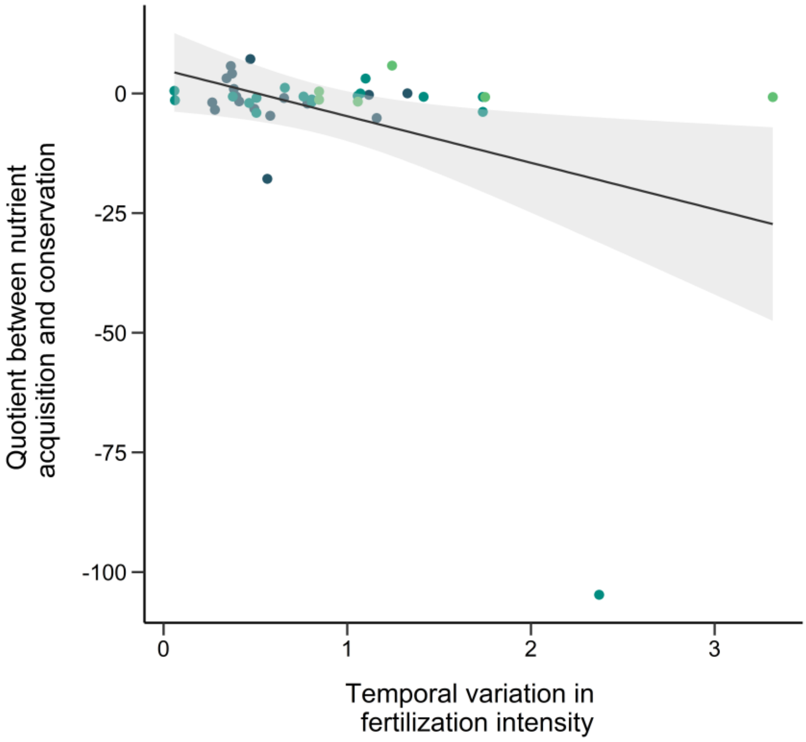


Figure S3: Relationship between the strength of the acquisition conservation trade-off at day 15 after fertilization in *Bromus hordeaceus* and temporal variation in fertilization intensity (F_{var}). Points represent population means of five to seven seed families (Schwäbische Alb in dark blue, Hainich in cyan, Schorfheide-Chorin in light green).

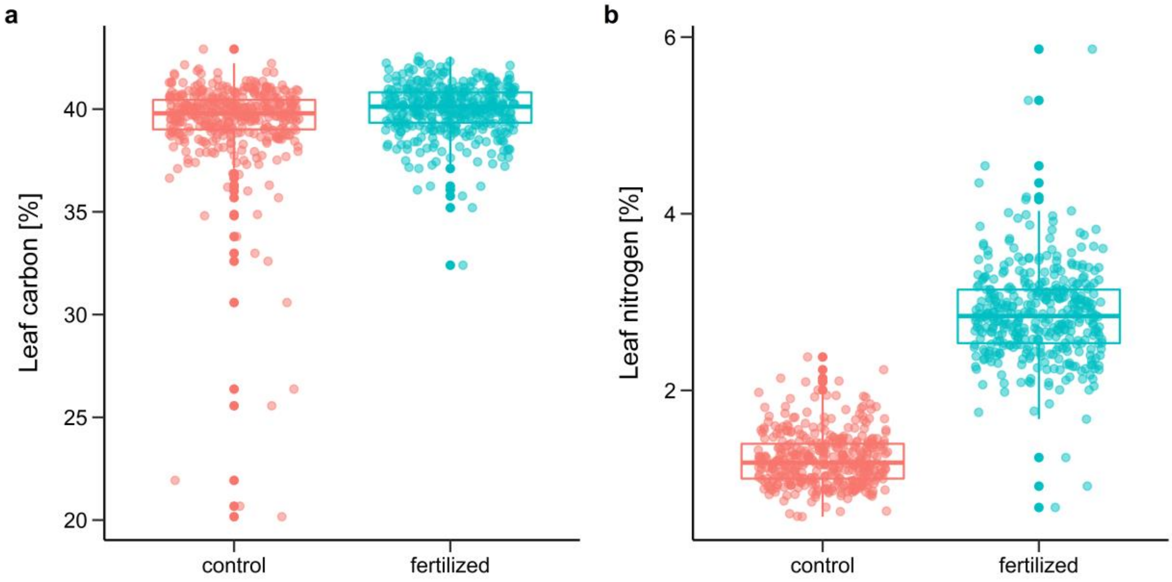


Figure S4: Box-plot comparing **a**) leaf carbon and **b**) leaf nitrogen of *Achillea millefolium* control plants (red) and fertilized plants (blue) two weeks after fertilization.

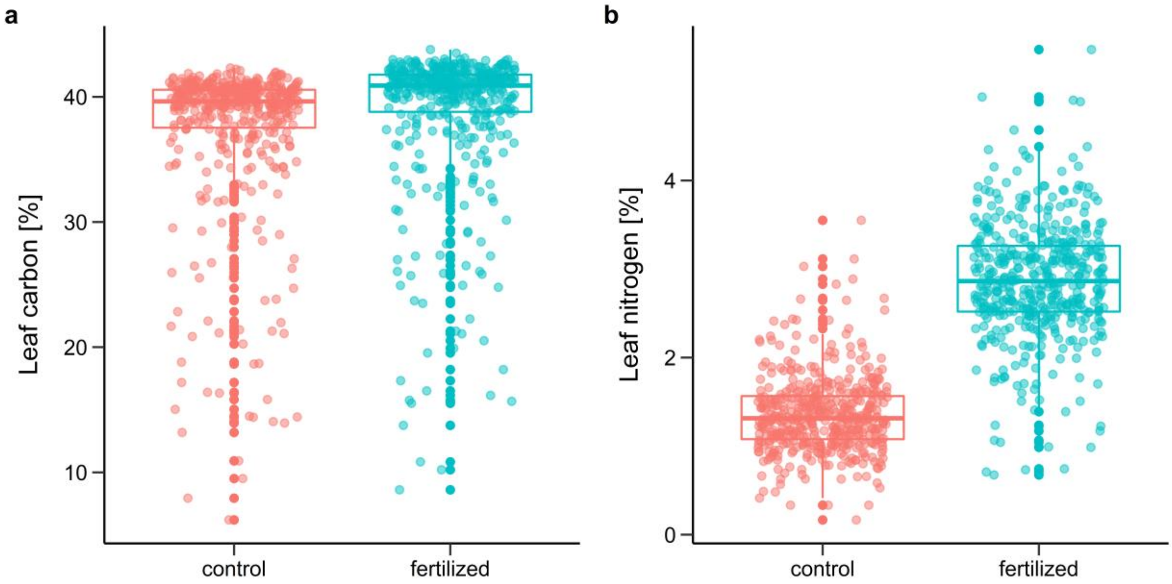


Figure S5: Box-plot comparing **a**) leaf carbon and **b**) leaf nitrogen of *Bromus hordeaceus* control plants (red) and fertilized plants (blue) two weeks after fertilization.

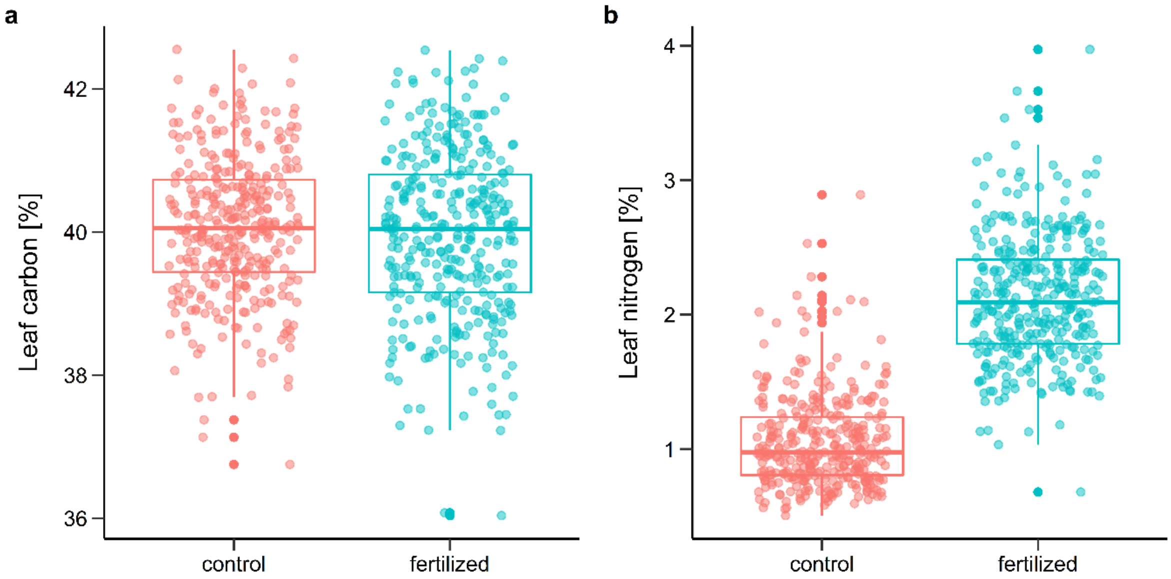


Figure S6: Box-plot comparing **a**) leaf carbon and **b**) leaf nitrogen of *Plantago lanceolata* control plants (red) and fertilized plants (blue) two weeks after fertilization.

Chapter II

Variation in regrowth ability in relation to land-use intensity in three common grassland herbs

Anna Kirschbaum, Oliver Bossdorf, JF Scheepens

Abstract

Aims

Plant populations in managed grasslands are subject to strong selection exerted by grazing, mowing and fertilization. Many previous studies showed that this can cause evolutionary changes in mean trait values, but little is known about the evolution of phenotypic plasticity in response to land use. In this study, we aimed to elucidate the relationships between phenotypic plasticity – specifically, regrowth ability after biomass removal – and the intensity of grassland management and levels of temporal variation therein.

Methods

We conducted an outdoor common garden experiment to test if plants from more intensively mown and grazed sites showed an increased ability to regrow after biomass removal. We worked with three common plant species from temperate European grasslands, with seed material from 58 – 68 populations along gradients of land-use intensity, ranging from extensive (only light grazing) to very intensive management (up to four cuts per year).

Important findings

In two out of three species, we found significant population differentiation in regrowth ability after clipping. While variation in regrowth ability was unrelated to the mean land-use intensity of populations of origin, we found a relationship with its temporal variation in *Plantago lanceolata*, where plants experiencing less variable environmental conditions over the last 11 years showed stronger regrowth in reproductive biomass after clipping. Thus, while mean grazing and mowing intensity may not select for regrowth ability, the temporal stability of the environmental heterogeneity created by land use may have caused its evolution in some species.

Keywords: environmental heterogeneity, grazing, inter-annual temporal variation, intraspecific variation, mowing, phenotypic plasticity

Introduction

Around 26 % of earth´s land surface is currently used as agricultural grasslands (Foley *et al.* 2011). A major aspect of environmental variation in these managed grasslands are the recurring disturbances exerted by mowing or grazing. Plants are sessile, and therefore, in order to survive, they need to be able to adapt to these disturbances. The effects of land-use intensity on the evolution of plant traits have received increasing attention during the last decades, and previous studies have repeatedly demonstrated genetically based trait changes in morphology, physiology and phenology in response to grassland management. For instance, grazing and mowing often select for dwarf morphology and prostrate growth (Warwick and Briggs 1979), tolerance to damage (Louault *et al.* 2005; Díaz *et al.* 2007), and phenological shifts (Reisch and Poschlod 2009; Völler *et al.* 2017). However, the possibility that plants could also adapt to grassland management through phenotypic plasticity has received less attention so far.

Common land-use practices not only change the means of environmental conditions, but they also create spatiotemporal heterogeneity (Suzuki 2008). Grazing and mowing in managed grasslands often vary in aspects such as timing, duration and frequency (Wang *et al.* 2017) and can create spatially and temporally heterogeneous conditions. Grazing, for example, creates temporal and spatial heterogeneity through selective grazing, trampling and patchy dung deposition, whereas mowing creates temporal heterogeneity in growing conditions (but increases spatial homogeneity). Increasing land-use intensity increases the heterogeneity of environmental conditions within one year (intra-annual). Additionally, if farmers change management practices between years, this creates additional environmental heterogeneity across years (inter-annual).

One mechanism for plants to respond to heterogeneous habitat conditions is through phenotypic plasticity (Schlichting and Levin 1986; Valladares *et al.* 2007), the ability of a genotype to produce multiple phenotypes depending on the environmental conditions (Bradshaw 1965). Hence, phenotypic plasticity may to some extent buffer against the effects of land-use practices. In addition, as a genetically controlled trait itself (Pigliucci 2005), phenotypic plasticity might also evolve when patterns of environmental heterogeneity (i.e. land-use intensity or inter-annual variation) differ among populations (Suzuki 2008). Generally, if phenotypic plasticity improves plant performance (i.e. fitness) across environments, it is adaptive and thus expected to evolve if genetic variation for plasticity exists (Relyea and Morin 2002; Van Kleunen and Fischer 2005; Pigliucci 2005). In heterogeneous environments, where plants need to rapidly adjust their morphology, physiology or reproduction to maintain or improve fitness, plasticity of functional traits should be adaptive and thus evolve (Matesanz *et al.* 2010; Gianoli and Valladares 2012; Scheiner 2013). Many empirical studies have demonstrated the importance of phenotypic plasticity for organisms to cope with environmental heterogeneity and global change (Matesanz *et al.* 2010). In homogeneous environments, in contrast, plasticity should not evolve, or should even be lost if greater plasticity is associated with fitness costs (Van Kleunen and Fischer 2005).

The ability of a plant to maintain relatively constant fitness under stressful conditions is called plant tolerance (Rejmánek 2000; Simms 2000; Barton 2013). In managed grasslands, a key tolerance trait is the ability to (partially) compensate for biomass loss through regrowth (Strauss and Agrawal 1999). Plants with a high tolerance to damage would show a rather flat reaction norm of fitness over a range of damage intensities (Rejmánek 2000; Simms 2000; Richards *et al.* 2006). This will likely be achieved through plasticity in some morphological or physiological traits that affect regrowth and thus fitness (Bradshaw 1965; Strauss and Agrawal 1999; Tiffin 2000), such as increased photosynthetic rates after stress (McNaughton 1979; Sultan *et al.* 1998; Strauss and Agrawal 1999) or the use of storage compounds for regrowth after damage (Oesterheld and McNaughton 1988; Strauss and Agrawal 1999).

So far, only few studies explored the extent of genetic variation in and evolution of plasticity of plants in relation to different intensities of grazing and mowing. Moreover, to our knowledge, no study has investigated plasticity in relation to temporal variation of management intensity. For instance, Carman & Briske (1985) found that regrowth after clipping was greater in plants from three grazed sites than in plants from three non-grazed sites, suggesting selection of increased regrowth ability under recurrent biomass removal. Likewise, Oesterheld & McNaughton (1988) found population differentiation in growth rate, tillering frequency and leaf morphology along a gradient of three grazing intensities in *Themeda triandra* in response to a clipping treatment. Other studies found no differences in plasticity between land-use origins. For instance, Rotundo and Aguiar (2007) studied three *Poa ligularis* populations with different grazing intensity and history and found no differences in their responses to clipping. Similarly, Suzuki (2008) showed that *Persicaria longiseta* plants from one grazed population did not respond differently to clipping than those from two ungrazed populations. Given such studies with contrasting results and low population replication, larger and better-replicated studies across multiple species are needed for more powerful tests of the effects of grassland management on the evolution of plasticity and its adaptive value.

So far, most previous studies of phenotypic plasticity in relation to grassland management compared few discrete, contrasting environments. This however, might be misleading as intermediate environmental states are not considered (Kreyling *et al.* 2018). To overcome this, one should look at plastic responses along a gradient of an environmental condition. In the 150 grassland plots of the Biodiversity Exploratories (see methods), grazing and mowing intensities have been quantified continuously since 2006. These plots are therefore ideally suited to investigate plant responses along a land-use gradient. As increasing management intensity represents increasing intra-annual environmental heterogeneity, we expect the strength of phenotypic plasticity to increase with increasing land-use intensity. Moreover, these land-use data from the Biodiversity Exploratories also allow to test for a relationship between inter-annual temporal variation in land use and phenotypic plasticity.

Here, we studied the evolution of regrowth ability in relation to grassland management in three temperate grassland plants. In a common garden experiment, we subjected plants from a broad range of land-use intensities to a standardized clipping treatment. Specifically, we asked the following questions: 1) Is there population differentiation for regrowth ability in the three studied plant species? If yes, is population-level variation in regrowth ability associated with 2) the mean grazing and mowing intensity in the studied populations or 3) with the inter-annual temporal variation in these land-use practices?

Material and methods

Study system

We worked in a system of grasslands plots located in three regions in Germany, embedded in the framework of the Biodiversity Exploratories, a large-scale and long-term project investigating relationships between land use, biodiversity and ecosystem functioning (https://www.biodiversity-exploratories.de). The three regions – the UNESCO Biosphere Reserve Schorfheide-Chorin, the Hainich National Park and surrounding areas, and the UNESCO Biosphere Area Schwäbische Alb – span a north-south transect in Germany and are approximately 300 km apart from each other. In each of the three regions, there are 50 grassland plots at distances of a few hundred meters to 30-40 km (mean distance 13.4 km) (Fischer *et al.* 2010; Völler *et al.* 2017). The grassland plots, each with an area of 50 × 50 m, cover a land-use gradient from extensive to very intensive management based on different types (grazing, mowing, fertilization) and intensities of land use. For each plot, annual inventories record the mowing intensity as the number of cuts per year, and grazing intensity as livestock units per hectare, multiplied with the grazing period and weighted by livestock type (cattle, sheep, horse, goat) (Blüthgen *et al.* 2012; Vogt *et al.* 2019). To integrate land-use history and inter-annual variation, we used a long-term measure of grazing and mowing intensity, separately calculated as the global mean for all three regions using the LUI calculation tool (Ostrowski *et al.* 2020) implemented in BExIS (http://doi.org/10.17616/R32P9Q), from 2006-2016, i.e. all available data before 2017, the year of our seed sampling. We used average intensity of grazing and mowing across 11 years as a proxy for intra-annual environmental heterogeneity, and we calculated temporal variation of grazing and mowing intensity as their standard deviation over 11 years as a proxy for inter-annual environmental heterogeneity.

Study species and seed collection

Between May and September 2017, we collected seeds from three common grassland species – *Achillea millefolium* L., *Plantago lanceolata* L., *Bromus hordeaceus* L. – from the grassland plots of the Biodiversity Exploratories. We selected the three species based on their frequent occurrence and high abundance in the plots. As the species differ in their timing of seed maturity, we visited all 150 grassland plots several times and collected ripe seeds from a maximum of 12 plant individuals on each plot where the species occurred. However, current land use, especially mowing, might have prevented sampling on every plot where the species occurred. We finally collected seeds of *A. millefolium* from 58 plots, of *B. hordeaceus* from 68 plots and of *P. lanceolata* from 63 plots (Table 1). We generally chose individuals randomly but with at least 1 m distance between each. In the remainder of this paper, the seeds from one individual are referred to as seed families and all individuals from one plot as a population.

We dried all seeds at room temperature and stored them in paper bags at 4°C in the dark until further use.

Common garden experiment

In April 2018 we sowed seeds from seven seed families per population per species in cultivation trays (PL, TK series, Pöppelmann GmbH & Co KG, Lohne) filled with a standard potting soil (Topferde CL T Classic, Einheitserdewerke, Sinntal-Altengronau) and placed them in a shade house at the experimental station of the University of Tübingen. In the case of *P. lanceolata*, we stratified seeds prior to germination for two weeks at 4°C in the dark. Three weeks (five weeks for *P. lanceolata*) after sowing, we transplanted two seedlings (one individual per pot) from each of five to seven seed families, depending on germination success, per population in 1L pots (Ø 13 cm, Hermann Meyer KG, Langenau) filled with a sand-soil-mixture (2:1:1, Rheinsand 0-2 mm, Flammer GmbH, Mössingen : Pro Start, Brill Substrate GmbH, Georgsdorf : Topferde CL T Classic, Einheitserdewerke, Sinntal-Altengronau) and added an equivalent of 60 kg N ha^{-1} of a NPK slow-release fertilizer (Osmocote Pro 5-6 M, 19-9-10 + 2MgO + SP, Herman Mayer KG, Langenau). We placed each pot on an individual saucer (Ø 15 cm, Herman Mayer KG, Langenau) in a fully randomized order on an experimental field (see Fig. S1) covered with weed-control fabric (PPX® 100 g/m^2 Ground Cover, Hermann Mayer KG, Langenau). After four weeks of growth, during which the plants were watered as needed but at least two times per week, we clipped half of the plants (one seedling per seed family) with pruning shears at the soil surface. After the clipping, all plants grew for another 16 weeks with the same watering regime. In September 2018, we harvested the aboveground biomass of all plants, separated it into reproductive and vegetative biomass, dried it for four days at 70°C and weighed all samples. Reproductive biomass was defined as flowering stems plus inflorescences for *P. lanceolata* and *B. hordeaceus*, and only inflorescences for *A. millefolium*.

Statistical analyses

To balance our data for the statistical analyses, we only included seed families where both clipped and unclipped plants had survived the experiment. Therefore, the sample sizes for the statistical analyses were smaller than those in the experiment (Table 1). To test for population differentiation in regrowth ability and treatment effects on plant performance, we fitted linear mixed effects models that included region, population, treatment and the interaction between population and treatment as fixed effects and seed family as a random effect. We included region to account for possible variation caused by the large-scale geographic variation among the three regions, but we included it as fixed effect because n = 3 is generally considered insufficient for estimating a random effect (Harrison *et al.* 2018).We analyzed each species separately, with total biomass – the sum of reproductive and vegetative biomass – as response variable for all three species, and reproductive biomass only for *P. lanceolata*. For the other two species, statistical analyses of reproductive biomass were impossible since not enough individuals flowered during our experiment.

Table 1: The three plant species used in our study, with some key life-history traits, the numbers of individuals and populations in the experiment and in the final statistical analyses, and land-use variation in their populations or origin. The mowing and grazing intensities are average numbers of mowing events and life-stock densities, respectively, across 11 years. Temporal variation in grazing and mowing is calculated as the standard deviation of each land-use factor, respectively, across 11 years.

	Achillea millefolium	*Bromus hordeaceus*	*Plantago lanceolata*
Plant family	Asteraceae	Poaceae	Plantaginaceae
Pollination type	Insects	Self/Wind	Wind
Breeding system	Obligate outcrosser	Predominantly autogamous	Obligate outcrosser
Life cycle	Perennial	Annual	Perennial
# Individuals (experiment/analyses)	812 / 736	952 / 832	882 / 772
# Populations (experiment/analyses)	58 / 58	68 / 67	63 / 63
Mowing intensities (# pop)	0 – 2.12 (48)	0 – 2.63 (57)	0 – 2.80 (54)
Grazing intensities (# pop)	0 – 7.33 (53)	0 – 7.33 (55)	0 – 4.31 (49)
Temporal variation in mowing	0 – 0.85	0 – 0.85	0 – 0.85
Temporal variation in grazing	0 – 3.57	0 – 3.24	0 – 3.57

To test for relationships of land use and regrowth ability, we first calculated an index of plasticity for each seed family as the log response ratio (LRR) of total biomass between clipped and unclipped plants. Because of zeroes in the reproductive biomass, we could not calculate LRRs of reproductive biomass for individual seed families in *P. lanceolata*, and we therefore first calculated the population means of clipped and unclipped plants and then the LRR of these two. Two populations of *P. lanceolata* where only clipped plants had reproduced were excluded from these analyses. To analyze the variation in LRR of total biomass, we fitted linear mixed models with region and land use as fixed effects and population as random effect. Land use in these models was either mowing intensity, or grazing intensity, or a compound variable of both, further called "total damage intensity", averaged across 11 years. Total damage intensity is calculated as the square-root of the sum of the standardized grazing and mowing intensities. Following the calculation of the land-use index by Blüthgen et al. (2012), we applied square-root transformation to minimize the effects of outliers and balance the distribution. The variation in LRR of reproductive biomass of *P. lanceolata* was analyzed with a simpler linear model with only region and land use as fixed effects.

In order to test for relationships between regrowth ability and temporal variation in land use, we first calculated the inter-annual variability of mowing and grazing intensity as the standard deviation of these land-use factors from 2006 to 2016, and we calculated temporal variation of total damage intensity as the square root of the sum of the standard deviations of both mowing and grazing from 2006 to 2016. As measure of temporal variation we preferred the SD over the coefficient of variation (CV) because we thought that in this case absolute amounts of damage were more biologically meaningful than relative ones (when using the CV), and because there were little problems with spurious correlations between SDs and means (see Discussion). To test for relationships between temporal variation and regrowth ability, we fitted linear mixed models with the LRR of total biomass as response variable, region and one of the measures of temporal variation in land use as fixed effects, and population as random effect. Again, the LRR of reproductive biomass of *P. lanceolata* was analyzed with simpler linear models that included only region and one of the measures of temporal variation in land use.

To ensure normality and homoscedasticity of model residuals we log-transformed total biomass and square-root-transformed reproductive biomass for all biomass analyses. For the analyses of LRR, residuals were generally normally distributed and homoscedastic without transformation.

All statistical analyses were done in R version 3.6.1 (R Core Team 2019), using in particular the *lme4* (Bates *et al.* 2015) and *lmerTest* (Kuznetsova *et al.* 2017) packages. When analyzing multiple species and land-use factors, we generally adjusted false discovery rates (FDR) following Benjamini and Hochberg (1995).

Results

Population differentiation

Populations of *A. millefolium*, *B. hordeaceus* and *P. lanceolata* significantly differed in their mean biomass (Table 2, Fig. 1). Moreover, populations of *A. millefolium* also differed in their regrowth response to the clipping treatment (population-by-treatment interaction in Table 2; Fig. 1a), suggesting genetically based variation in regrowth ability among the studied populations. In contrast, we found no significant population by treatment interactions for *B. hordeaceus* and *P. lanceolata* (Table 2, Fig. 1b and c), indicating a lack of population differentiation in regrowth ability. However, populations of *P. lanceolata* differed significantly in their production of reproductive biomass as well as in the responses of their reproductive biomass to the clipping treatment (Table 2, Fig. 1d), indicating genetic differentiation among populations in regrowth ability with regard to this trait.

Regrowth ability in response to land use

Regrowth ability in response to clipping, estimated as the log response ratio (LRR) of total biomass, or as the LRR of reproductive biomass in *P. lanceolata*, was uncorrelated to mowing intensity, grazing intensity, and total damage intensity in all three studied species (Table 3). The LRRs of total biomass were also unrelated to temporal variation of land use, but we found that the LRR of *P. lanceolata* reproductive biomass significantly correlated with inter-annual temporal variation in mowing intensity (Table 3). Under temporally more variable mowing regimes, the LRR of *P. lanceolata* reproductive biomass was significantly lower, i.e. populations were responding less plastically to the clipping treatment in our experiment (Fig. 2).

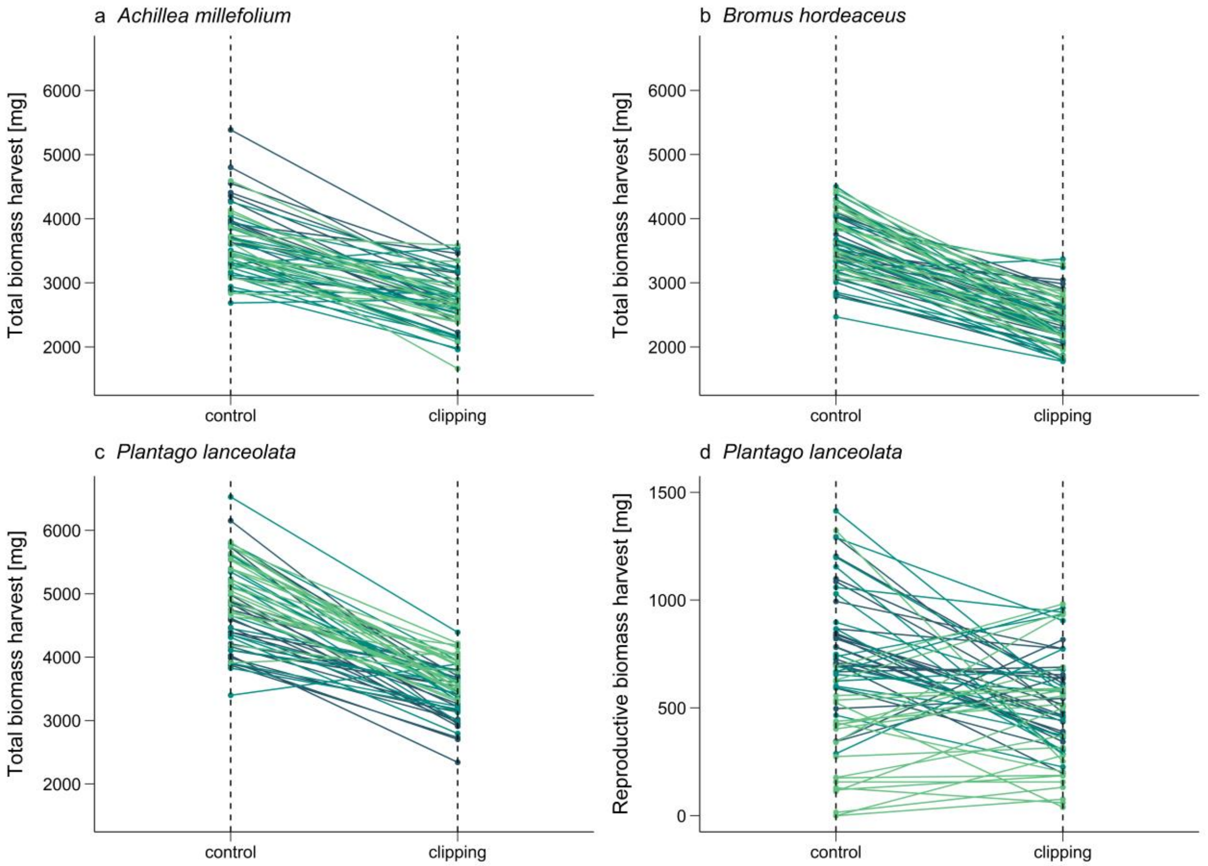


Figure 1: Reaction norms for total biomass of *Achillea millefolium* **a**), *Bromus hordeaceus* **b**) and *Plantago lanceolata* **c**) and for reproductive biomass of *Plantago lanceolata* **d**) in response to clipping (Schwäbische Alb in dark blue, Hainich in cyan, Schorfheide-Chorin in light green). Each reaction norm represents the mean values of five to seven seed families per population.

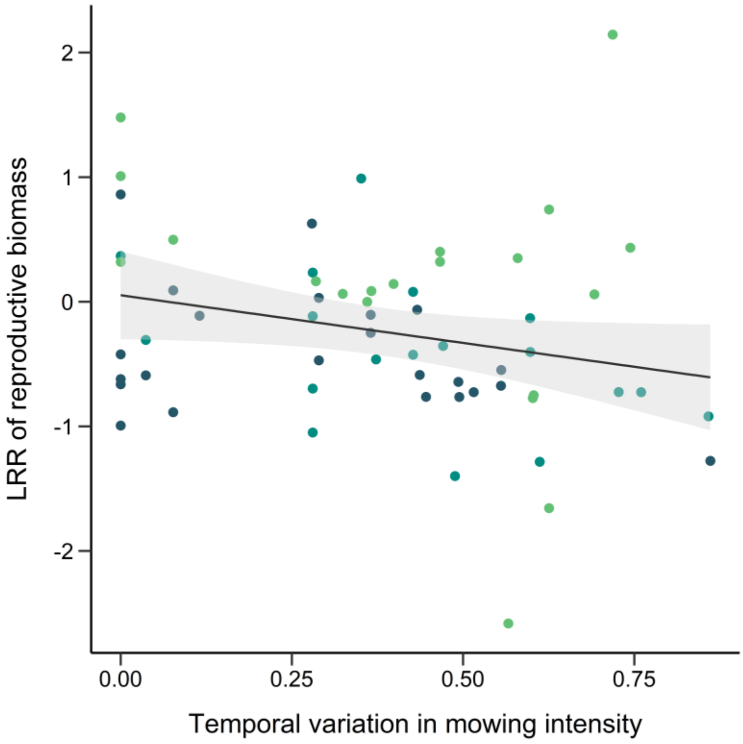


Figure 2: Relationship between the temporal land-use variation, calculated as the standard deviation of mowing frequencies across 11 years, and the plastic regrowth ability of 61 *Plantago lanceolata* populations, quantified as the log response ratio (LRR) of their reproductive biomass to experimental clipping (Schwäbische Alb in dark blue, Hainich in cyan, Schorfheide-Chorin in light green).

Table 2: Results of linear mixed effects models testing effects of region of origin, population, clipping treatment and the interaction of the latter two on the total biomass of *Achillea millefolium*, *Bromus hordeaceus* and *Plantago lanceolata* as well as the reproductive biomass of *Plantago lanceolata*. Bold *P*-values indicate effects that are significant ($P < 0.05$) after FDR correction. df = degrees of freedom.

	Achillea millefolium Total biomass				*Bromus hordeaceus* Total biomass				*Plantago lanceolata* Total biomass				*Plantago lanceolata* Reprod. biomass			
	Num df	Den df	*F*-value	*P*-value	Num df	Den df	*F*-value	*P*-value	Num df	Den df	*F*-value	*P*-value	Num df	Den df	*F*-value	*P*-value
Region	2	596	2.55	0.079	2	682	0.11	0.897	2	634	1.60	0.202	2	615	10.68	**<0.001**
Population	55	309	2.26	**<0.001**	64	348	1.89	**<0.001**	60	323	1.53	**0.011**	60	323	1.90	**<0.001**
Treatment	1	308	284.02	**<0.001**	1	347	414.46	**<0.001**	1	322	338.86	**<0.001**	1	323	6.67	**0.010**
Pop:Treat	57	308	1.37	**0.049**	66	347	0.83	0.823	62	322	1.04	0.414	62	322	1.95	**<0.001**

Table 3: Results of linear mixed effects models testing for relationships between land-use intensities (mowing, grazing, or both processes combined) and their temporal variation, and the regrowth abilities, i.e. phenotypic plasticity in response to clipping, of *Achillea millefolium*, *Bromus hordeaceus* and *Plantago lanceolata*. We estimated regrowth ability as the log response ratios (LRR) of total biomass for all three species, and as the LRR of reproductive biomass for *Plantago lanceolata*. All land-use intensities and their temporal variation are based on 11 years of data (2006-2016), with temporal variation calculated as the standard deviations of mowing and grazing intensity, respectively. Bold *P*-values indicate values significant (*P*<0.05) after FDR correction. df = degrees of freedom.

		Achillea millefolium LRR of total biomass			*Bromus hordeaceus* LRR of total biomass			*Plantago lanceolata* LRR of total biomass			*Plantago lanceolata* LRR of reprod. biomass	
	Num df	Den df	*F*-value	*P*-value	Den df	*F*-value	*P*-value	Den df	*F*-value	*P*-value	*F*-value	*P*-value
Region	2	54	1.80	0.175	400	1.91	0.149	376	1.06	0.349	3.51	0.036
Mowing intensity	1	55	0.10	0.753	400	0.82	0.365	376	0.07	0.791	0.05	0.817
Region	2	54	1.96	0.150	400	1.80	0.167	376	1.34	0.263	3.53	0.036
Grazing intensity	1	53	0.45	0.503	400	0.72	0.397	376	0.54	0.463	0.34	0.562
Region	2	54	1.85	0.167	400	1.49	0.226	376	1.20	0.301	3.52	0.036
Total damage intensity	1	53	0.46	0.500	400	0.24	0.621	376	0.40	0.529	0.23	0.631
Region	2	53	1.80	0.175	400	1.33	0.288	376	1.23	0.293	3.92	0.026
Temporal variation in mowing intensity	1	56	0.20	0.653	400	0.45	0.480	376	0.99	0.320	6.63	0.013
Region	2	54	2.50	0.091	400	1.77	0.172	376	1.40	0.248	3.55	0.035
Temporal variation in grazing intensity	1	52	2.75	0.103	400	0.66	0.419	376	0.81	0.369	0.60	0.442
Region	2	54	2.36	0.105	400	1.73	0.179	376	1.80	0.167	3.71	0.031
Temporal variation in total damage intensity	1	52	1.48	0.230	400	0.41	0.523	376	1.91	0.168	3.27	0.076

Discussion

Because of their recurring mowing and grazing, managed grasslands constitute spatially and temporally heterogeneous environments, to which plants should adapt through evolution of phenotypic plasticity. To test this, we studied population differentiation in clipping responses in three common grassland plants, across a large number of grasslands differing in the intensity and temporal variation of their management. We found significant population variation in clipping responses in *Achillea millefolium*, but this variation was unrelated to land use. In *Plantago lanceolata*, however, regrowth ability in reproductive biomass was negatively correlated with the temporal variation of mowing intensity, indicating that evolution of this type of plasticity may be favored only if the factors that create heterogeneity are relatively stable across years.

Population differentiation in regrowth ability

We found that grassland populations of *A. millefolium* exhibited significant population differentiation in regrowth of total biomass in response to clipping. However, the total biomass responses of *B. hordeaceus* and *P. lanceolata* did not differ among populations, indicating a lack of genetically-based variation in regrowth ability in these two species. In contrast to the lack of variation in regrowth ability of total biomass, we found significant population differences in regrowth of reproductive biomass in *P. lanceolata*. Comparable previous studies found mixed results: Bergelson and Crawley (1992) also found population differences in *Ipomopsis aggregata* responses to a clipping treatment, and Damhoureyeh and Hartnett (2002) demonstrated that populations of three tallgrass prairie species differed in their root/shoot ratio and reproductive allocation responses to clipping. In another study, however, three populations of *Persicaria longiseta* responded similarly to a clipping treatment in several traits, suggesting a lack of variation in plasticity in this system (Suzuki 2008).

The contrasting findings among our three study species might result from differences in pollination type and breeding system. The observation of genetic variation for plasticity in reproductive biomass of *P. lanceolata* is rather unexpected, as the species is an obligate outcrosser and wind-pollinated. Together, these factors should lead to strong gene flow between populations and thus reduce the potential for population differentiation as shown for the total biomass response of *P. lanceolata*. However, several previous studies, including some from the same study regions, already demonstrated population differentiation in this species. Comparing *P. lanceolata* plants from a late- and early-mown meadow and a pasture, van Tienderen and van der Toorn (1991) showed local adaptation for seed yield, onset of flowering and growth habit. Additionally, Gáspár et al. (2019) found significant genetic and epigenetic differentiation among populations of *P. lanceolata* from the Biodiversity Exploratories. Thus, genetic and phenotypic differentiation in geographically close populations of *P. lanceolata* are possible and may indeed reflect adaptation to local environmental conditions. In contrast, geographically restricted insect pollination and short seed dispersal distances in *A. millefolium* limit gene flow and might have favored the observed population differentiation. In *B.*

hordeaceus, a predominately selfing species, population differentiation in mean traits were previously found in response to grazing and mowing within the Biodiversity Exploratories (Völler *et al.* 2013, 2017). Thus, as *B. hordeaceus* is already adapted to grazing and mowing by other means, this might have prevented the further evolution of population differentiation in regrowth ability.

Regrowth ability and mean land use intensities

Although we found population differentiation in regrowth ability of *A. millefolium* and *P. lanceolata* after biomass removal, there were no relationships between this variation and the mean mowing or grazing intensities of the populations of origin. This is in contrast to our prediction that increasing land-use intensity would result in the evolution of increased regrowth ability. A similar lack of relationship was found by Suzuki (2008) in *Persicaria longiseta* which, after a long history of grazing, exhibited adaptation in mean values of fitness-related traits, but no evolution of the response to clipping in these traits. In contrast, comparing long-term grazed or mown populations of *Gentianella campestris* with such that were unmanaged, increased regrowth ability evolved only in managed habitats (Lennartsson *et al.* 1997), suggesting that land use positively selects for this trait. Likewise, population differentiation in grazing tolerance in response to clipping was related to long-term grazing history in three tallgrass prairie species (Damhoureyeh and Hartnett 2002).

In our experiment, management intensities varied from no mowing at all to around three times mowing per year and from no grazing to a year-round permanent pasture (averaged across 11 years), i.e. our study populations encompassed a very broad range of management intensities in these types of temperate grasslands. However, looking at total damage, there are no populations that are not subject to recurring biomass removal, through either grazing or mowing. Although we are missing a true zero-point, we would have expected that the strength of the land-use gradient and the heterogeneous conditions this creates within a single growing season would have exerted a selective pressure strong enough to affect regrowth ability of our study species differentially.

One possible explanation could be that the 11 years of land-use data that we based our study on might not sufficiently reflect the longer-term management history of these populations, and that in such cases the time period of 10+ years might not represent the relevant evolutionary time scale for the studied plants. Indeed, several of the aforementioned studies that found relationships between clipping tolerance and land use worked on sites where grazing history was known for at least 25 years (Damhoureyeh and Hartnett 2002) or even several hundred years (Lennartsson *et al.* 1997), which likely represent adequate time frames for such evolutionary changes. Since we do not know the land use histories of our study sites before the recording started in 2006, we cannot rule out, that in some there were significant changes. Most of the studied grasslands have certainly been grasslands for a much longer time, but it is possible e.g. that some higher management intensity plots were previously less intensively managed, or vice versa. Nevertheless, we did find significant population variation in regrowth ability for some traits and species, so these differences must have been driven by other factors such as soil fertility (Leiss and Müller-Schärer 2001) or result from random genetic drift in populations with limited gene flow (Mitchell-Olds *et al.* 2007).

Regrowth ability and inter-annual land-use variation

Grassland management regimes might change across years, for instance when farmers alter the frequency of mowing or the stocking densities on pastures, creating not only heterogeneous environmental conditions within one year but also temporal variation in land use across years. We calculated temporal variation in mowing intensity as the standard deviation (SD) of mowing intensity across 11 years. We preferred SDs over the popular coefficient of variation (CV) because we thought that especially for mowing the absolute change matters and represents a similar perturbance, irrespective of the mean mowing intensity. The SD is of course less independent from the mean than the CV, so one needs to consider the possibility of spurious correlations, i.e. a relationship between SD and regrowth could be a side-effect of one between the mean and regrowth. However, since we did not find any association between mean mowing intensity and regrowth ability in the first place, and the correlation between the mean and SD of mowing intensity was rather weak (Pearsons´s r: 0.36), we did not consider this a problem in our case.

We expected that regrowth ability of plant populations would increase with increasing temporal variation, but to our surprise, we found the opposite in one of the study species: regrowth ability for reproductive biomass of *P. lanceolata* was higher under temporally less variable mowing conditions (with the coefficient of variation in mowing intensity this pattern would not be visible; see Fig. S2). Generally, plasticity is expected to evolve under heterogeneous environmental conditions (Scheiner 2013), but it is thought to be more advantageous in more predictable environments as plants need to be able to accurately forecast future conditions to benefit from plasticity (Scheiner 1993; Stuefer 1996; Alpert and Simms 2002; Lande 2009; Reed *et al.* 2010). In our study system, low temporal variation in mowing intensity means that farmers maintained the same numbers of cuts per year over the 11 years compared to high temporal variation where mowing intensity changed across years. Hence, a temporally less variable mowing regime represents a heterogeneous but more stable environment. While the importance of environmental stability for the evolution of plasticity has been demonstrated in theoretical models (Scheiner 1993; Jong 1999; Lande 2009), empirical tests remain scarce. In one of the few existing studies, plastic responses in allocation and fitness traits to nutrient stress were strongest in *Hordeum spontaneum* plants from a more stable Mediterranean habitat characterized by low inter-annual variation in precipitation (Volis *et al.* 2002). Altogether, theoretical and empirical results, including those from our study, suggest that heterogeneous but stable environmental conditions may be required for the evolution of plasticity.

We found a relationship between regrowth ability and the temporal variation of mowing but not grazing intensity. A possible explanation for this could be the reliability of environmental cues in these two land-use processes. Mowing usually affects the whole population equally and creates spatially homogeneous environmental conditions. As it affects the whole population, it might be a reliable cue for initializing regrowth. Grazing, in contrast, is spatially patchy and does not affect all plant individuals in a population equally (Völler *et al.* 2017) but usually creates spatial heterogeneity and might therefore be less stable at the population level. In our study, only temporal variation in mowing intensity, but not grazing intensity, was related to differential regrowth ability. Hence, less stable environmental conditions, either within one year or across several years could hamper selection on regrowth ability in response to a land-use gradient. Furthermore, in our study system some grassland plots are managed with a combination of both mowing and grazing, which might have added complexity, as the different cues might interfere with each other and create unreliable conditions. Altogether, we propose that temporal stability and reliability of recurring biomass removal events may be key factors determining whether population differentiation in regrowth ability will evolve in response to land use, because only reliable cues allow to infer future environmental conditions from current ones (Reed *et al.* 2010).

We only found a relationship between the temporal variation of mowing intensity and reproductive biomass but not with the total biomass of *P. lanceolata* or any of the other two species. This could reflect the closer proximity of reproductive biomass to true plant fitness, which selection acts on. Losing unripe seeds, which represent a substantial investment of plant resources, in a mowing or grazing event might be much more critical for plant success than losing only a part of the vegetative biomass. Unfortunately, the other two study species flowered too infrequently to also analyze their reproductive output, and to test whether the results from *P. lanceolata* also hold for them. *B. hordeaceus* is an annual species, and we initially expected it to complete its life-cycle fastest. However, our experiment apparently did not provide the minimal conditions for the species to complete its life cycle, indicating a discrepancy between field and experimental conditions, which may also have affected responses to clipping.

Conclusions

Land use in grasslands creates environmental heterogeneity, which should affect the evolution of phenotypic plasticity in plants. So far, to our knowledge, no previous study explored the effects of inter- and intra-annual heterogeneity in land use on the evolution of plasticity. We studied plasticity in response to biomass removal in three common grassland plants from 58 – 68 populations and found genetic variation in regrowth ability in two of the three species. While land-use intensity was unrelated to variation in regrowth ability, we found that inter-annual temporal variation in mowing was significantly related to regrowth ability in one of the studied species. Thus, our data suggest that inter- rather than intra-annual variation affected the evolution of regrowth ability in this system. We need multi-species common garden comparisons of the resident populations and better fitness measures to test this hypothesis more broadly.

Funding

The work was supported by the DFG Priority Program 1374 "Infrastructure-Biodiversity-Exploratories" through project SCHE 1899/1-1 to JFS.

Acknowledgments

We thank the managers of the three Exploratories, Kirsten Reichel-Jung, Iris Steitz, Sandra Weithmann, Juliane Vogt and Miriam Teuscher, and all former managers for their work in maintaining the plot and project infrastructure; Christiane Fischer for giving support through the central office, Andreas Ostrowski for managing the central data base, and Markus Fischer, Eduard Linsenmair, Dominik Hessenmöller, Daniel Prati, Ingo Schöning, François Buscot, Ernst-Detlef Schulze, Wolfgang W. Weisser and the late Elisabeth Kalko for their role in setting up the Biodiversity Exploratories project. The work has been funded by the DFG Priority Program 1374 "Infrastructure-Biodiversity-Exploratories" through project SCHE 1899/1-1 to JFS. Field work permits were issued by the responsible state environmental offices of Baden-Württemberg, Thüringen, and Brandenburg.

Supplements

Figure S1: Common garden at the University of Tübingen.

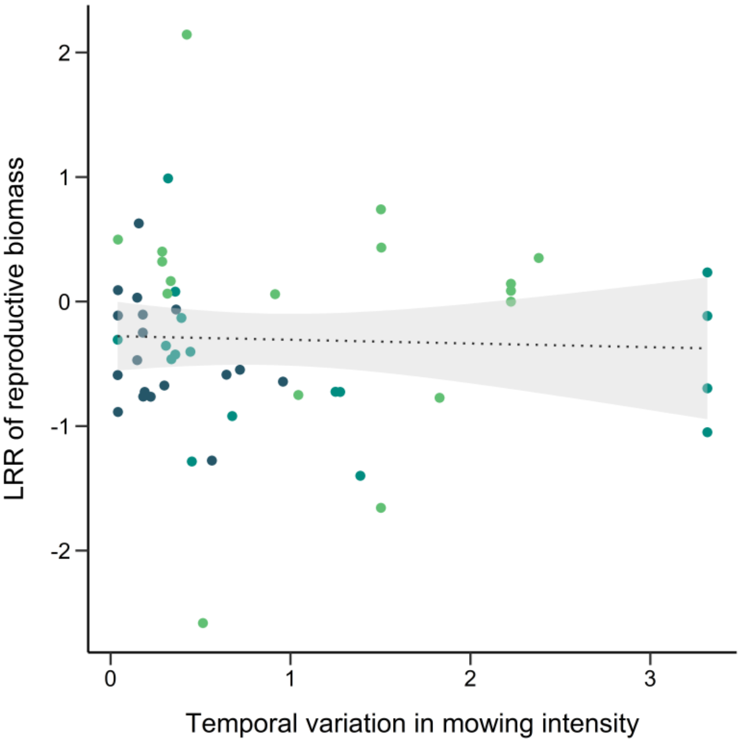


Figure S2: Relationship between the inter-annual variation in mowing intensity across 11 years, here calculated as the coefficient of variation (CV), and the plastic regrowth ability of 52 *Plantago lanceolata* populations, with color codes as in Fig. 2. Results of a linear model with region of origin and CV of mowing intensity: $F_{region} = 1.92$, $P_{region} = 0.157$, $F_{CV\_M} = 0.51$, $P_{CV\_M} = 0.479$.

Chapter III

Storage of non-structural carbohydrates is genetically variable in grassland populations of *Plantago lanceolata* but not related to land-use intensity or plant regrowth ability

Anna Kirschbaum, Günter Hoch, Oliver Bossdorf, JF Scheepens

Abstract

Aims

Non-structural carbohydrates (NSCs) are important storage reserves of plants, and they may play a key role in their ability to recover from disturbance events such as drought, fire or biomass removal. In managed grasslands, plants regularly experience aboveground biomass removal by grazing or mowing. If NSCs influence plant tolerances to these damages, then land-use intensification could lead to adaptive changes in NSC storage allocation.

Methods

In a common garden experiment we quantified NSC storage in the taproots of *Plantago lanceolata* plants from 63 grassland populations that covered a broad range of land use intensities, and we tested if pre-clipping levels of NSCs were related to land-use intensity and the regrowth ability of plants after aboveground biomass removal.

Important findings

We found significant genetic variation in NSC storage among populations, but in contrast to our expectation NSC storage correlated negatively with mowing intensity and its temporal variation. Moreover, NSC variation was unrelated to the regrowth ability of *Plantago lanceolata* after aboveground biomass removal, which suggests that this common grassland plant is not C-limited. Our results indicate that NSCs do not affect regrowth ability of *Plantago lanceolata* on a long-term perspective, thus future research should address short-term effects of NSCs.

Keywords: common garden, disturbance tolerance, genetic variation, grassland management, grazing, mowing, NSC

Introduction

In order to grow and maintain metabolic functions plants assimilate carbon through photosynthesis. If photosynthesis does not match the actual carbon demand of a plant, assimilated carbon can be stored in the form of non-structural carbohydrates (NSCs) in stems, leaves, and roots as well as in specialized organs such as storage roots (Janeček and Klimešová 2014; Martínez-Vilalta *et al.* 2016) for later use. Depending on the plant species, NSCs include low-molecular weight sugars (fructose, sucrose, glucose) and starch (in some herbs and grasses also fructans) (Chapin *et al.* 1990; Martínez-Vilalta *et al.* 2016; Landhäusser *et al.* 2018). Starch that is stored during the day is used to maintain growth and metabolism during the night (Smith and Stitt 2007). However, plants can also mobilize stored carbon during episodes of low carbon availability that would otherwise result in net carbon depletion and decreased photosynthetic activity, e.g. during spring regrowth (Heilmeier *et al.* 1986), flowering and fruit production (Horibata *et al.* 2007), periods of abiotic stress such as drought (Hartmann *et al.* 2013), or during regrowth after biomass removal (Greub and Wedin 1971; Richards and Caldwell 1985; Li *et al.* 2002; Carpenter *et al.* 2008).

In temperate European grasslands, plants are usually subjected to regular aboveground biomass removal through grazing or mowing. These management practices remove much of the photosynthetically active tissue, and plants need to regrow. The ability of plants to compensate for a loss of biomass, which is related to the concept of grazing tolerance (McNaughton 1983), is not only based on the production of new leaves (Richards and Caldwell 1985; Visser *et al.* 1997; Morvan-Bertrand *et al.* 1999), but often also on the mobilization of stored carbon reserves (NSC) that enhance the recovery of photosynthetically active tissue (Morvan-Bertrand *et al.* 1999; Schnyder and de Visser 1999). Several previous studies showed that stored carbohydrates are indeed mobilized from storage tissue after defoliation and translocated to newly produced shoots (Danckwerts and Gordon 1987; Morvan-Bertrand *et al.* 1999; Schnyder and de Visser 1999). The abilities of building up carbon reserves and quickly mobilizing them after biomass loss therefore seem crucial for plant fitness and should thus be selected for.

In managed grasslands, the frequency of grazing or mowing, as well as the intensity of grazing in terms of the duration and type of animals, can vary considerably among different grasslands. If there is genetic differentiation in NSC storage among grasslands that results from natural selection, then we would expect adaptation to management intensity in three main ways: (1) in the amount of carbon stored before the start of management in spring, (2) in the degree of carbon mobilization after disturbance, and (3) in the replenishment of carbon reserves after disturbance. A few previous studies showed that NSC storage of plants can indeed adapt to the severity of biomass loss (Palacio *et al.* 2012; Benot *et al.* 2019). For instance, after natural defoliation of *Pinus nigra* by the pine processionary moth, NSC content in needles and stems decreased but replenished proportional to defoliation intensity in just one growing season, with more heavily defoliated trees re-accumulating more NSC (Palacio *et al.* 2012). Moreover, Benot et al. (2019) showed that cattle grazing intensity influenced early-season NSC content in five grass species, with intensely grazed plants showing higher NSC concentrations than moderately grazed ones. In addition, all plants replenished NSC storage at the end of the grazing period to pre-grazing levels irrespective of the grazing intensity (Benot *et al.* 2019). All of these studies indicate that biomass loss, through natural defoliation or land use, may exert selection on NSC storage patterns.

In contrast to the influence of biomass removal on NSC storage, the influence of stored NSC on grazing tolerance in terms of regrowth has gained much less attention. So far, no consensus exists about whether higher NSC concentrations before biomass removal increase regrowth after disturbance (Davies 1965; Richards and Caldwell 1985; Hogg and Lieffers 1991). For example, a study on NSCs and regrowth of *Lolium perenne* found that only fructans influenced early regrowth, whereas at later stages there was no relationship between pre-defoliation levels of NSC and regrowth anymore (Morvan-Bertrand *et al.* 1999). In contrast, *Populus* saplings compensated better when cut in late fall, when root starch content is high, compared to saplings cut in spring, when root starch content is lower (Landhäusser and Lieffers 2002). Also, the saplings cut in late fall replenished their root starch to pre-disturbance levels, while spring-cut saplings achieved only 20% recovery (Landhäusser and Lieffers 2002).

While the research described above has demonstrated links between biomass removal and NSC storage, so far no study has, to our knowledge, tested for population differentiation in NSC storage, i.e. demonstrated that NSC storage differences between populations are heritable, and related to the intensity and/or timing of grassland management. We also know that grassland plants show phenotypic adaptations towards variation in grassland management, for instance through prostrate growth forms (Warwick and Briggs 1979; Díaz *et al.* 2007), phenological escape strategies (Völler *et al.* 2013, 2017) or increased grazing tolerance (Rosenthal and Kotanen 1994; Louault *et al.* 2005; Díaz *et al.* 2007). Hence, we hypothesize that variation in biomass removal through grazing and/or mowing should also select for different levels of NSC storage.

Here, we tested for population differentiation in NSC storage along a gradient of land-use intensity, specifically grazing and mowing intensities, in *Plantago lanceolata*. This short-lived perennial is widespread in the northern hemisphere and stores NSC mainly in its taproot. The most important carbohydrates for this species are raffinose-family oligosaccharides, sorbitol and glucose (Janeček *et al.* 2011). NSC content in *P. lanceolata* decreases after defoliation (Lee *et al.* 2015) and re-accumulation of total NSCs appears to be higher in damaged than undamaged plants under nutrient-rich conditions (Latzel, Janeček, Doležal, *et al.* 2014). We also tested if pre-clipping levels of NSC affected the regrowth ability of *P. lanceolata*. For this, we used data from another common garden experiment, conducted with the same populations of *P. lanceolata*, in which we tested the effects of land-use intensity on the regrowth ability of *P. lanceolata* after a clipping treatment (Kirschbaum *et al.* 2021).

Specifically, we were interested in the following questions: 1) Is there genetic variation in the concentration of non-structural carbohydrates (NSCs) in *P. lanceolata* taproots along a land-use gradient? 2) Does NSC storage in *P. lanceolata* correlate with land-use intensity, in particular with grazing and mowing? 3) Do pre-defoliation levels of NSC explain variation in regrowth ability in *P. lanceolata*?

Methods

Study area

Our study was conducted within the framework of the Biodiversity Exploratories, a large-scale and long-term project investigating relationships between land use, biodiversity and ecosystem processes. The project consists of a network of study sites in three regions of Germany – the UNESCO Biosphere Reserve Schorfheide-Chorin, the Hainich National Park and surrounding areas, and the UNESCO Biosphere Area Schwäbische Alb – with 50 grassland plots (50 × 50 m) in each region. Together, the three regions span a north-south transect of about 800 km in Germany, and the distances between plots within each region range from less than a km to about 30 km, with a mean distance of 13.4 km. All grasslands are continuously managed; they cover broad land-use gradients from unfertilized and lightly grazed grasslands to strongly fertilized meadows and pastures that are heavily mown or grazed several times per year. In each plot, the type and intensity of management are monitored annually (Vogt *et al.* 2019), and intensity of management is calculated using the LUI calculation tool (Ostrowski *et al.* 2020) implemented in BExIS (http://doi.org/10.17616/R32P9Q), as follows: (1) fertilization intensity as the amount of nitrogen applied per hectare (kg N ha^{-1}), (2) grazing intensity as the units of livestock per hectare, multiplied with grazing period and weighted by type of livestock (horse, cattle, sheep, and goat), and (3) mowing intensity as the number of cuts per year (Blüthgen *et al.* 2012). In our study, we used 11 years of monitoring data (2006 – 2016) to calculate average land-use intensities for all plots.

Seed material

Between May and September 2017 we collected seeds of *Plantago lanceolata* from all plots in the Biodiversity Exploratories where the species occurred. Depending on seed maturity (influenced by land-use management), we visited all plots up to three times and collected ripe seeds of 12 individuals per plot. Below, we refer to these as seed families (= all seeds from one individual) in contrast to populations (= all individuals from one plot). The 12 individuals were selected randomly but with at least 1 m distance between each. We were able to collect seed material from 63 plots. We dried all seeds at room temperature in paper bags and subsequently stored them in the dark at 4°C until further use.

Common garden experiment

To investigate heritable variation in NSC content in the taproots of *P. lanceolata* we used the same seedlings as in an outdoor common garden experiment conducted from April-October 2018 in Tübingen (Kirschbaum *et al.* 2021). Depending on germination success, we used one seedling from five to seven seed families per population, altogether 370 plants. The procedures for sowing and germination (cold stratification), pots (1 L), soil type (sand-soil mixture), fertilization (equivalent of 60 kg N ha^{-1}), watering (*ad libitum* but at least twice per week) and random placement on the field site were identical to the aforementioned common garden experiment (for details see Kirschbaum *et al*. 2021). Six weeks after transplanting the seedlings to pots and placing them outside in our experimental garden, we recorded if plants flowered, measured rosette diameter as a proxy for plant size, and harvested belowground biomass for NSC analysis.

Non-structural carbohydrate analysis

As we were only interested in the taproot, we removed all secondary roots with scissors and scalpels. The morphology of the taproot – with the largest diameter at the top and narrowing downwards – allowed us to define the total length of the taproot as the point where the diameter became less than 1 mm. The processed and cleaned taproots were kept on ice until later, on the same day, when we heated the taproots in a microwave oven at 900 W for two times 30 s with cooling of 5 s in between the heating repetitions, to stop NSC-modifying enzymes. After that, we dried the roots for three days at 60°C and subsequently weighed them. We ground the dried root material in metal grinding jars (10 mL, stainless steel, Retsch GmbH, Haan) with two grinding balls (Ø 7 mm, stainless steel, Retsch GmbH, Haan) in a mixer mill (MM 400, Retsch GmbH, Haan) at 20 Hz until all material was pulverized. We then transferred the ground root material into 2 mL Eppendorf tubes (Eppendorf AG, Hamburg) and stored them over silica gel until further use.

To estimate non-structural carbohydrates (NSCs), defined as free, low molecular weight sugars (glucose, fructose, sucrose) plus starch, we employed a slightly modified protocol by Landhäusser et al. (2018). After heating approximately 15 mg of grinded root sample with distilled water, the soluble fraction was treated with invertase (from baker's yeast; Grade VII, ≥300 U/mg, I4504-1G, Sigma-Aldrich Corp, Saint Louis, Missouri) and isomerase (from baker´s yeast; Type III, ammonium sulfate suspension, ≥400 U/mg, P5381-5KU, Sigma-Aldrich Corp., Saint Louis, Missouri) to digest fructose and sucrose to glucose (see protocol S1 in Landhäusser *et al.* 2018). The glucose was then quantified by spectrophotometry after enzymatic conversion to gluconate-6-phosphate (see protocol S4 in Landhäusser *et al.* 2018). The insoluble starch fraction was treated with α-amylase (from *Bacillus licheniformis*; lyophilized powder, 500 – 1500 U/mg, A4551-100mg, Sigma-Aldrich Corp., Saint Louis, Missouri) and amyloglucosidase (from *Aspergillus niger*; ROAMYGLL – 3500 U, 6 U/mg, Roche Diagnostics GmbH, Mannheim) to convert the starch into glucose (see protocol S2 in Landhäusser *et al.* 2018), which was then quantified as above.

Statistical analysis

To obtain a measure of absolute NSC content per plant, we calculated NSC content as the sum of sugar and starch content multiplied by the dried root weight. We then divided this measure of absolute NSC content by the rosette diameter of that plant to obtain a measure of NSC content relative to plant size, henceforth termed relative NSC content. We calculated relative sugar and relative starch contents in the same manner. To test for population differentiation in relative NSC content, we fitted a linear model with relative NSC content as response variable and region of origin and population nested within regions as fixed effects. Since some plants had started flowering before our harvest, we included flowering as a binary covariate (yes - 1/no - 0) in our model.

To investigate the effects of land-use intensity on relative NSC contents we fitted linear mixed-effects models with flowering (binary), region of origin and one of three land-use factors – grazing, mowing or a combination of both variables, further referred to as total damage, calculated as the square-root of their summed standardized values – as fixed effects and population as random effect. To test for the effects of inter-annual temporal variation in land-use intensity we further calculated the standard deviations of mowing and grazing intensity over eleven years (2006-2016), and for total damage the square root of the sum of the two standard deviations. We applied linear mixed-effects models with relative NSC content as response variable, flowering (binary), region of origin and one of the measures of temporal land-use variation as fixed effects, and population as random effect. We repeated the same analyses separately for relative sugar and relative starch content. The statistical assumptions of normality and homoscedasticity of residuals were achieved through a log-transformation of the response variable in all linear mixed effects models. Since our analyses of the different NSC measures and land-use factors constituted multiple testing, we used the Benjamini-Hochberg (Benjamini and Hochberg 1995) correction for false discovery rates.

Finally, we tested if relative NSC content was related to the regrowth ability of *P. lanceolata* after a clipping treatment. For this we used data from a common garden experiment (Kirschbaum *et al*. 2021) with the same plant material where we had previously tested for plasticity of regrowth ability after a clipping treatment, and had calculated plasticity as the log response ratio of clipped versus unclipped plants in terms of their total and reproductive biomass at the end of the growing season (for details see Kirschbaum *et al*. 2021). We fitted linear mixed-effects models with plasticity of total biomass of *P. lanceolata* as response variable, region of origin and flowering-corrected residuals of relative NSC content as fixed effects, and population as random effect. Additionally, we fitted a linear model with the plasticity of reproductive biomass of *P. lanceolata* as a response variable, and region of origin and flowering-corrected residuals of relative NSC content as explanatory variables. We used population-level plasticity values of reproductive biomass, as plasticity calculations at the seed family level yielded infinite values because most plants did not reproduce, and hence there was no need for including population as a random factor. We repeated the same analyses for relative sugar and relative starch content. In all models, the assumptions of normality and homoscedasticity of residuals were met without transformation of the response variable.

The statistical analyses described above were done with R version 3.6.1 (R Core Team 2019), the *lme4* package (Bates *et al.* 2015) and the *lmerTest* package (Kuznetsova *et al.* 2017).

Results

The relative NSC content of *P. lanceolata* differed significantly among regions of origin and populations (Figure 1, Table 1), and the patterns were similar for relative sugar and relative starch content (Table 1). We also found that all three carbohydrate variables were strongly influenced by whether a plant had been flowering during the experiment or not.

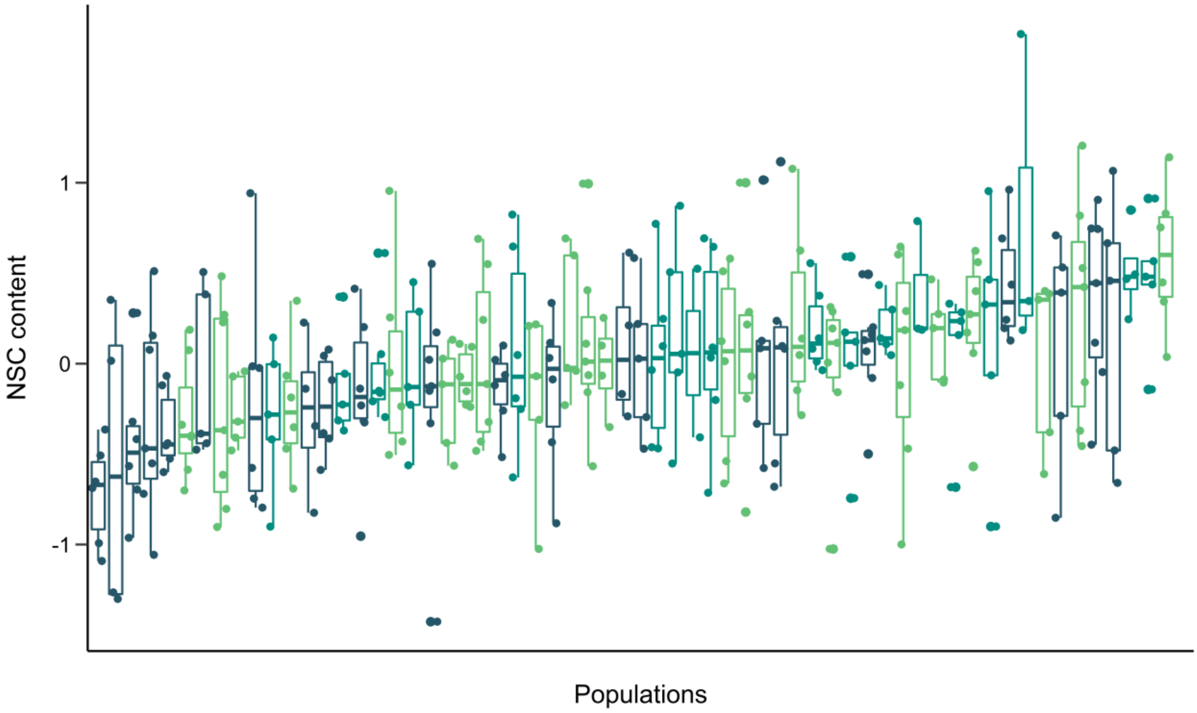


Figure 1: Variation in the content of non-structural carbohydrates (NSCs) among 63 grassland populations of *Plantago lanceolata*. The values are flowering-corrected residuals of relative NSC content (= absolute NSC content divided by rosette diameter). The boxplots are ordered by their medians, and colored by region of origin (Schwäbische Alb in dark blue, Hainich in cyan, Schorfheide-Chorin in light green).

We found a significant negative relationship between the mowing intensity of the grasslands of origin and relative NSC content of the *P. lanceolata* plants (Table 2, Figure 2), but there were no relationships with sugar or starch content, or with the other two measures of mean management intensity (Table 2). The pattern was similar for temporal variation in land-use: the temporal variation in mowing intensity was negatively related to relative NSC as well as relative sugar content (Table 2, Figure 3), but we found no relationships with the other two measures of temporal land-use variation.

The plasticity in regrowth ability of both total biomass and reproductive biomass of *P. lanceolata* biomass was unrelated to relative NSC, sugar or starch content (Table 3).

Table 1: Results of linear models testing the effects of flowering state (y/n), region of origin and population on relative content of sugar, starch and total non-structural carbohydrates (NSC) as the sum of both, in *Plantago lanceolata*. Bold *P*-values are significant (< 0.05) after FDR correction. df = degrees of freedom.

	Sugar content			Starch content			Total NSC content		
	df	*F*-value	*P*-value	df	*F*-value	*P*-value	df	*F*-value	*P*-value
Flowering	1	5.70	**0.018**	1	16.70	**<0.001**	1	1.85	**0.004**
Region	2	7.46	**<0.001**	2	5.33	**0.005**	2	1.50	**0.002**
Population	59	1.50	**0.016**	59	1.39	**0.042**	59	0.34	**0.018**
Residuals	290			281			281		

Table 2: Results of linear mixed-effects models that test for relationships between land-use intensity (mowing intensity, grazing intensity, or total damage as the sum of the both), or its temporal variation, and stored non-structural carbohydrates (relative content (= absolute content divided by rosette diameter) of sugar, starch and total non-structural carbohydrates, the sum of both sugar and starch) in *Plantago lanceolata*. Each model includes flowering (y/n) as a binomial covariate, region as a fixed effect, and population of origin as a random effect. Bold *P*-values are significant (< 0.05) after FDR correction. df = degrees of freedom.

		Sugar content		Starch content		Total NSC content	
	df	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Flowering	1	1.33	0.250	6.96	**0.009**	2.66	0.104
Region	2	2.79	0.069	2.13	0.127	2.51	0.088
Mowing intensity	1	6.48	**0.014**	3.95	0.052	6.86	**0.011**
Flowering	2	1.25	0.265	11.40	**<0.001**	2.54	0.112
Region	1	4.70	**0.012**	1.09	0.343	4.36	**0.017**
Grazing intensity	1	0.50	0.498	0.96	0.332	0.62	0.434
Flowering	1	1.14	0.286	11.17	**<0.001**	2.39	0.123
Region	1	4.93	**0.010**	1.91	0.155	4.58	**0.014**
Total damage	2	2.22	0.142	0.24	0.626	1.93	0.170
Flowering	1	1.10	0.294	6.64	**0.010**	2.35	0.127
Region	2	7.14	**0.002**	4.46	**0.015**	6.33	**0.003**
Temporal variation in mowing intensity	1	8.13	**0.006**	1.27	0.265	6.41	**0.014**
Flowering	2	1.19	0.276	6.64	**0.010**	2.44	0.120
Region	1	4.92	**0.010**	3.53	0.035	4.5	**0.015**
Temporal variation in grazing intensity	1	0.04	0.850	1.11	0.298	0.2	0.658
Flowering	1	1.22	0.271	6.74	**0.010**	2.48	0.117
Region	2	5.34	**0.007**	3.28	0.044	4.71	**0.012**
Temporal variation in total damage	1	0.46	0.503	0.33	0.569	0.12	0.728

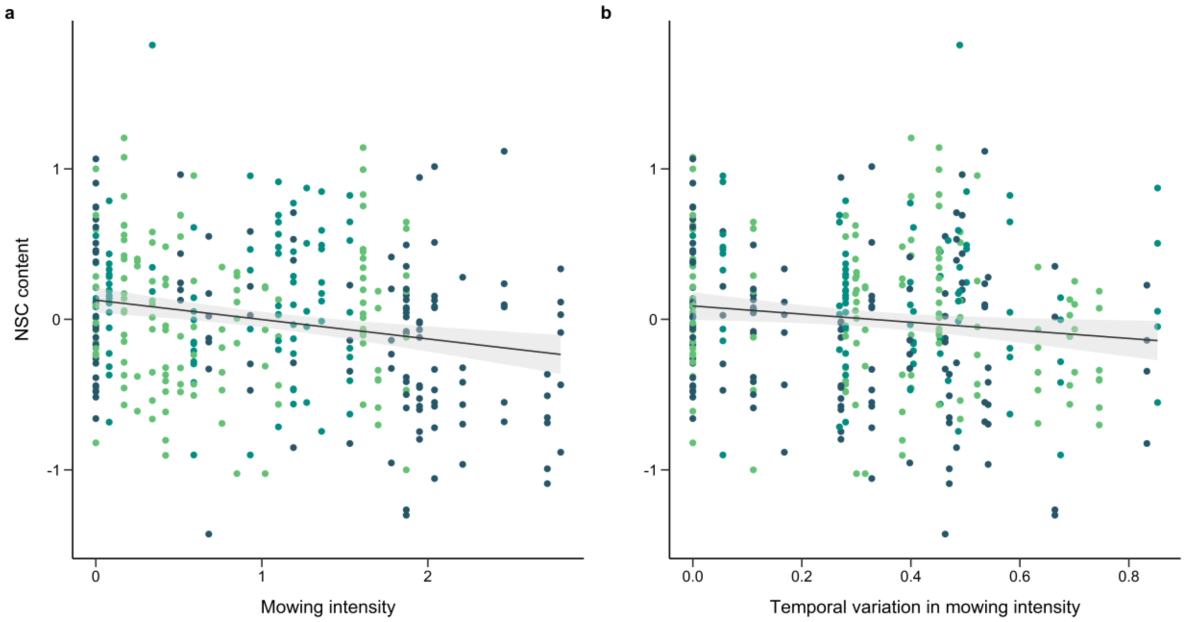


Figure 2: Relationship between **a**) mowing intensity, calculated as cuts per year averaged over eleven years (2006-2016), and **b**) temporal variation in mowing intensity (standard deviation of the number of cuts during 2006-2016) and the content of non-structural carbohydrates (NSC) of *Plantago lanceolata* in grasslands of different land use intensity. The values are flowering-corrected residuals of relative NSC content (= absolute NSC content divided by rosette diameter). Points represent individual values (Schwäbische Alb in dark blue, Hainich in cyan, Schorfheide-Chorin in light green).

Table 3: Results of linear mixed-effects models testing for relationships between contents of relative (= absolute content divided by rosette diameter) sugar, starch and total non-structural carbohydrates (NSC), the sum of both sugar and starch, of 63 grassland populations of *Plantago lanceolata* and their phenotypic plasticity of total or reproductive biomass in response to experimental clipping. Each model includes region of origin as a fixed effect and population of origin as a random effect. The plasticity data is from Kirschbaum *et al.* (2021). None of the *P*-values is significant after FDR correction. df= degrees of freedom.

		Plasticity of total biomass		Plasticity of reproductive biomass	
	df	*F*-value	*P*-value	*F*-value	*P*-value
Region	2	0.94	0.391	3.38	0.041
Sugar content	1	0.15	0.701	0.00	0.998
Region	2	0.90	0.408	3.44	0.039
Starch content	1	1.03	0.310	0.95	0.334
Region	2	1.11	0.331	3.39	0.041
Total NSC content	1	0.00	0.966	0.13	0.724

Discussion

The ability of plants to store non-structural carbohydrates (NSC) – low-molecular weight sugars and starch – can buffer the effects of environmental variability and disturbances such as drought, fire or herbivory, because it allows plants to mobilize stored NSCs during periods of carbon shortage. Here, we show that NSC storage of 63 populations of *Plantago lanceolata* in a common garden from along a land-use gradient exhibits genetically based population differentiation, with the amount of NSC storage negatively related to mowing but not grazing intensity of the populations of origin. The negative direction of the observed correlation was unexpected since we expected plants to invest more in NSC storage with increasing land-use intensity. It is thus possible that NSC storage does not play an important role for *Plantago lanceolata* recovery after disturbance. This idea is supported by the fact that levels of NSC were also uncorrelated to the regrowth ability of *P. lanceolata* after damage tested in another experiment.

Genetic variation in NSC storage

Generally, after accounting for the variance explained by flowering and region of origin, values of relative NSC, sugar and starch content still exhibited genetically based variation among populations. It is rather surprising that we found population differentiation in NSC storage as *P. lanceolata* is a wind-pollinated obligate outcrosser. This should lead to high rates of gene flow restraining the potential for population differentiation. However, Gáspár et al. (2019) also found genetic and epigenetic variation among an overlapping set of *P. lanceolata* populations. Moreover, levels of carbohydrate storage in *P. lanceolata* plants were found to be higher in mown plots than in abandoned plots, already indicating the potential of population differentiation of NSC in *P. lanceolata* (Janeček and Klimešová 2014). Thus, as phenotypic and genetic population differentiation among nearby populations of *P. lanceolata* varying in management is possible, the differences in NSC storage in this study may as well reflect local adaptation.

Effects of land use

When relating our measures of NSC storage to land-use intensity, we found that only mowing intensity and temporal variation in mowing intensity could explain variation in NSC and sugar concentrations. Plants originating from more intensively mown plots as well as plants experiencing a more variable mowing regime across years showed decreased NSC and sugar content. The negative relationship with mowing intensity is driven by populations from the Schwäbische Alb that show a broader range of mowing intensities compared to the sets of populations from the other regions (Fig. S1). In contrast, starch did not relate with mowing intensity or temporal variation therein.

As mentioned above, we expected plants from more intensively managed populations to store more NSC to be able to recover from recurrent damage through grazing or mowing. Since the study by Benot et al. (2019) additionally showed mostly positive correlations of storage compounds with grazing intensity, the patterns found in relation to land use in this study are rather ambiguous and unexpected, although our results clearly suggest that mowing intensity modulates storage patterns. In contrast to our findings, Benot et al. (2019) showed that in five grazing tolerant grass species (*Agrostis stolonifera*, *Cynosurus cristatus*, *Hordeum secalinum, Lolium perenne* and *Poa trivialis*) fructan and sucrose concentrations were higher under intensive grazing compared to moderate grazing before the start of the grazing season, suggesting an adaptation of NSC to grazing intensity. However, similar to our findings on NSC and sugar, they found that starch concentrations before the start of the grazing season rather declined with increasing grazing intensity. A similar pattern was found in a woody species, where after 11 years of repeated defoliations through insect herbivory, *Pinus nigra* trees exhibited higher NSC accumulation in sapwood compared to a non-defoliated control group (Palacio *et al.* 2012). Although these patterns may result from plastic adjustments, they suggest a similar ecologically useful response to cope with biomass removal.

One possible explanation for decreased NSC content under higher mowing intensity in our study might be that plants from more intensively mown plots do not invest that much in reserve storage but rather invest in aboveground biomass. This is because investment in storage compounds only makes ecological sense when it allows the plant to wait until the optimal moment to invest these compounds in aboveground biomass, such as directly after a mowing event. With frequent mowing, the time between several mowing events becomes too short such that investment from storage compounds into aboveground growth would not pay off anymore. In this scenario, storage might not be the optimal strategy and continuous investment in aboveground structures could be more advantageous.

Interestingly, we only found correlations of NSC storage with mowing intensity and not with grazing intensity. We think that the fact that mowing is much more predictable and homogeneous than grazing could explain this pattern. Grazing in contrast is heterogeneous in several aspects such as trampling by livestock, N deposition (animal dung) and probability in patterns of aboveground biomass removal leading to more unpredictable conditions. This in turn means that plants under grazing cannot anticipate when the next biomass removal by animals will occur. Hence, adaptation of NSC storage seems to be more advantageous under a mowing than under a grazing regime.

The importance of predictability in the adaptation of carbon storage to grassland management is likewise suggested by the negative relationship between relative NSC and sugar concentrations and temporal variation in mowing intensity. Temporal variation in mowing intensity describes how predictable a certain mowing regime was over a period of eleven years before the year of the seed sampling, with high levels representing unpredictable management. We found that under a more constant mowing regime *P. lanceolata* stores initially more NSC compared to a more variable mowing regime. Similar to grazing, plants under an unpredictable mowing regime cannot anticipate when the next mowing event will take place. Thus, results suggest that in unpredictable conditions it is better to invest in aboveground biomass than in storage.

It may be that, rather than pre-defoliation NSC levels, replenishment after disturbance is adapted to land-use intensity. As increasing grazing and mowing intensity mean shorter recovery phases for plants, faster and/or higher replenishment of NSC stores could be adaptive as well. For example, *Lolium perenne* plants that had been depleted of their storage carbon through repeated defoliations showed increased capacity for synthesis of water soluble sugars on day 1 after the last defoliation, suggesting an adjustment to repetitive carbon depletion in replenishment of storage reserves (Lee *et al.* 2010). Additionally, carbon replenishment in needles of *Pinus nigra* was proportional to insect herbivory intensity, with more strongly defoliated trees having higher levels of NSC at the end of the growing season, suggesting a short-term acclimation in the trees' NSC household (Palacio *et al.* 2012). By directly investigating replenishment capacity of NSCs in *P. lanceolata*, Lee et al. (2015) found pre-defoliation levels of NSC after 5 weeks of regrowth. These studies highlight the importance of NSC replenishment after biomass removal and its adaptation to disturbance intensity as further biomass removal, such as under high grazing and mowing intensity, during replenishment could be detrimental for subsequent plant growth and ultimately decrease total yield (Turner *et al.* 2006; Lee *et al.* 2010, 2015).

Regrowth ability

Investigating the role of pre-clipping levels of NSCs for regrowth ability after total aboveground biomass removal, we did not find a relationship with compensatory growth (Kirschbaum *et al.* 2021) neither in total biomass nor in reproductive biomass of *P. lanceolata*. However, the role of NSCs for the ability to regrow after damage is still subject of debate. Where some studies found a link between the mobilization and pre-defoliation levels of carbon reserves and regrowth ability (Hume 1991; Danckwerts 1993; Donaghy and Fulkerson 1998; Turner *et al.* 2006; Palacio *et al.* 2012) others found no association (Ryle and Powell 1975; Richards and Caldwell 1985). For example, *Lolium perenne* plants with higher levels of water soluble carbohydrates (WSC) before defoliation showed higher grazing tolerance in several traits such as leaf extension rate, dry matter yield as well as root survival (Donaghy and Fulkerson 1998). Additionally, because of earlier tiller initiation (i.e. production of photosynthetic active tissue) WSC replenishment started 4 days earlier in plants with higher pre-defoliation WSC levels (Donaghy and Fulkerson 1998). This, however, emphasizes that stored carbohydrates are only important for a short period after defoliation and that subsequent assimilation through photosynthesis becomes the main factor of carbon supply (Richards and Caldwell 1985; Hoogesteger and Karlsson 1992; Donaghy and Fulkerson 1997; Morvan-Bertrand *et al.* 1999; Lee *et al.* 2015). In several studies it has been shown that the mobilization of carbon reserves is indeed highest between day 1 and day 7 post defoliation and decreases shortly after again (Danckwerts and Gordon 1987; Visser *et al.* 1997; Morvan-Bertrand *et al.* 1999; Schnyder and de Visser 1999). For example, in perennial ryegrass, NSC influenced early regrowth but after 28 days the correlation between pre-defoliation NSC levels and leaf dry matter ceased (Morvan-Bertrand *et al.* 1999). This suggests that the effect of pre-defoliation NSC levels on regrowth diminishes over time and regrowth might become dependent on environmental factors affecting photosynthetic capacity. As regrowth ability in the aforementioned clipping experiment (Kirschbaum *et al.* 2021) was evaluated 16 weeks after clipping, this could have influenced our findings in such a way that potential early correlations between pre-clipping NSC levels and the ability to regrow disappeared over time. Moreover, NSCs may not be the only carbon compounds stored and potentially used for regrowth. Neutral lipids, together with starch and fructans exclusively synthesized for storage, and probably hemicellulose contribute to the carbon storage pool and might as well contribute to regrowth ability (Hoch *et al.* 2003; Schädel *et al.* 2010; Hoch 2015). Additionally, soluble sugars, as a fraction of NSCs, serve many different functions in a plant's metabolism besides growth and respiration. They are osmotically active, contribute to turgor maintenance and phloem transport, and they are involved in signaling and in cold tolerance (Morgan 1984; Gibson 2005; Krasensky and Jonak 2012). This however, makes it difficult to disentangle the direct effects of NSC to regrowth from other functions and may be partly responsible for the absence of relationships between NSCs and land use in our study. In addition, regrowth ability might also be dependent on other resources such as nitrogen (Wise and Abrahamson 2007; Latzel, Janeček, Hájek, *et al.* 2014; Erbilgin *et al.* 2014) or carbon (Hoogesteger and Karlsson 1992; Baptist *et al.* 2013). Yet, in our experiment, N-supply may not be a limiting factor for regrowth as we provided the plants with resource-rich and benign growing conditions. Moreover, the range of compensatory responses in the aforementioned clipping experiment (data from Kirschbaum *et al.* 2021) ranges from undercompensation to slight overcompensation and we did not find an association between pre-clipping NSCs levels and regrowth, we think that the studied *P. lanceolata* populations are not C-limited in the long-term. As N- and C-supply probably did not limit the ability to regrow after defoliation in this study, this might imply that other constraints limiting compensation through regrowth after aboveground biomass loss might exist.

Conclusion

In this study, we investigated genetic variation in the storage of non-structural carbohydrates (NSC) among 63 populations of *Plantago lanceolata*, its relationship with land-use intensity and the effects of pre-clipping NSC levels on regrowth ability. We found genetically based variation in NSC stored in the taproot of *P. lanceolata* among the investigated populations along a land-use gradient, with decreasing NSC storage along a mowing gradient and along a gradient of temporal variation in mowing intensity. Grazing intensity, however, did not affect NSC storage patterns, potentially because of its more heterogeneous character. Additionally, we could not find a link between pre-clipping NSC levels and regrowth ability after a defoliation treatment, which could have been obscured by rapidly recovered photosynthetic activity. This could indicate that *P. lanceolata* from these populations is not C-limited and that the ability to store and mobilize NSC after biomass removal might not be subject to evolution by natural selection. However, as we did find genetically based differences in NSC among populations, this suggests that carbon storage might be adapted to environmental conditions other than land use.

Acknowledgments

We thank the managers of the three Exploratories, Kirsten Reichel-Jung, Iris Steitz, and Sandra Weithmann, Juliane Vogt, Miriam Teuscher and all former managers for their work in maintaining the plot and project infrastructure; Christiane Fischer for giving support through the central office, Andreas Ostrowski for managing the central data base, and Markus Fischer, Eduard Linsenmair, Dominik Hessenmöller, Daniel Prati, Ingo Schöning, François Buscot, Ernst-Detlef Schulze, Wolfgang W. Weisser and the late Elisabeth Kalko for their role in setting up the Biodiversity Exploratories project. Our work has been funded by the DFG Priority Program 1374 "Infrastructure-Biodiversity-Exploratories" through project SCHE 1899/1-1 to JFS. Field work permits were issued by the responsible state environmental offices of Baden-Württemberg, Thüringen, and Brandenburg.

Supplements

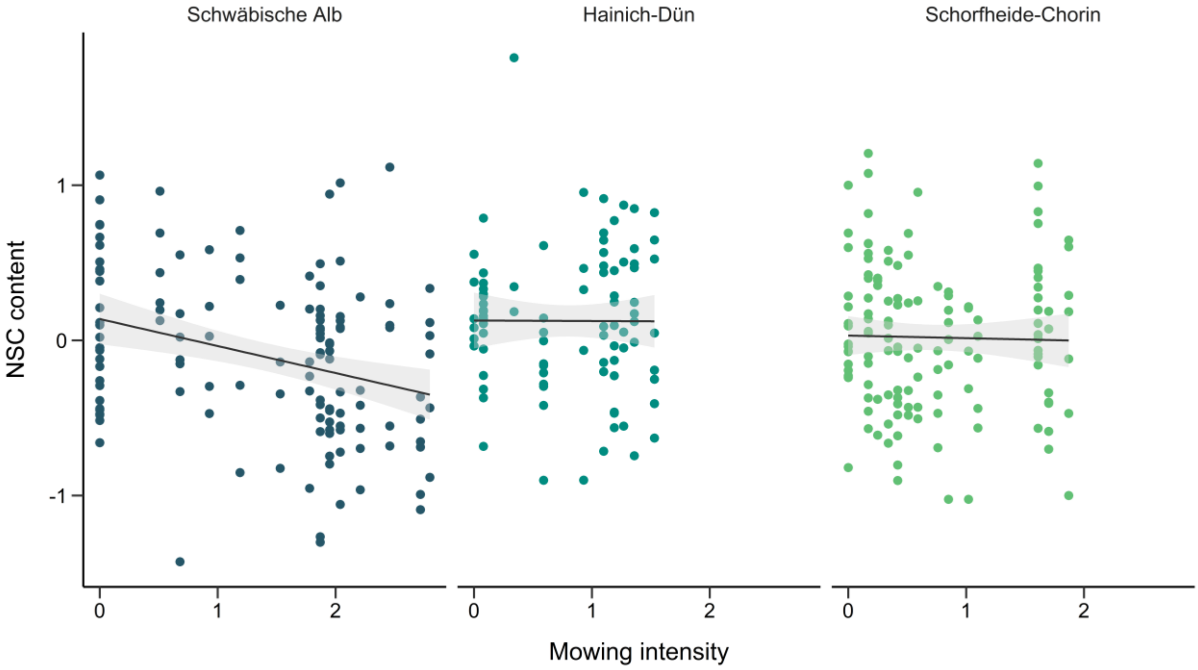


Figure S1: Relationships between mowing intensity, calculated as cuts per year averaged over eleven years (2006-2016), and the content of non-structural carbohydrates (NSC) of *Plantago lanceolata* in grasslands of different land use intensity. The values are flowering-corrected residuals of relative NSC content (= absolute NSC content divided by rosette diameter). Points represent individual values (Schwäbische Alb in dark blue, Hainich in cyan, Schorfheide-Chorin in light green).

Synthesis

Land-use change is currently characterized as the strongest global-change driver challenging the adaptive potential of animal and plant species worldwide. Especially plants, as sessile organisms with limited potential for migration, need to adapt in order to survive. Such evolutionary processes can occur over short periods of time, even within a few generations (Carroll *et al.* 2007). However, when changes in environmental conditions appear over shorter time periods and create heterogeneous conditions within a plants' life cycle, adaptation of trait means might not be sufficient. In such scenarios, the evolution of phenotypic plasticity, the ability of an individual to change its phenotype depending on environmental conditions, increases habitat niche breadth and as such tolerance to changing environmental conditions. However, studies on the evolution of plasticity in a land-use context remain rather scarce, compare only a few coarse land-use categories and are limited in their spatial extent as well as in sample size (Warwick and Briggs 1979; Carman and Briske 1985; Suzuki 2008).

In this thesis, I studied how land-use intensity and temporal variation in land-use intensity affected the evolution of phenotypic plasticity within three common grassland plants. In order to include many populations from a realistic background of grassland management with precise knowledge on land-use type and intensity, covering a broad geographic range, I took advantage of the network of grassland sites of the Biodiversity Exploratories (Fischer *et al.* 2010; Blüthgen *et al.* 2012; Vogt *et al.* 2019). I used seeds from 58 – 69 populations of *Achillea millefolium*, *Bromus hordeaceus* and *Plantago lanceolata* from the sites of the Biodiversity Exploratories to conduct two common garden experiments with treatments to investigate if increasing land-use intensity selected for increasing strength of phenotypic plasticity. In **Chapter I**, I studied the opportunistic response of the three species to increased nutrient availability in terms of aboveground biomass, leaf nitrogen and chlorophyll content. In **Chapter II**, I investigated regrowth ability – a homeostatic response – after removal of aboveground biomass. Additionally, in **Chapter III**, I looked at the relationship between non-structural carbohydrates and regrowth ability of *P. lanceolata*. Below, I shortly summarize the results from my studies under the aspects of 1) population differentiation of plastic responses, 2) the association of plasticity with mean land-use intensity, 3) the association of plasticity with temporal variation in land-use intensity and 4) the role of non-structural carbohydrates (NSCs) in regrowth ability of *P. lanceolata*. I will further discuss the implications of my results for the understanding of the evolution of phenotypic plasticity in a land-use context, identify remaining questions and consider the role of land use in the preservation of intraspecific biodiversity.

Population differentiation

Strength and direction of phenotypic plasticity may differ not only among species, but due to evolutionary forces such as genetic drift or adaptation to different environmental conditions also among populations within the same species (Schlichting 1986; Schlichting and Levin 1990; Sultan 1995; Leiss and Müller-Schärer 2001; Berg *et al.* 2005). As land-use type and intensity often differ among grassland sites and even among neighboring grasslands, differentiation in plastic responses of functional traits among populations is expected. I indeed found differences in plastic responses, both towards increased nutrient availability (**Chapter I**) and aboveground biomass removal (**Chapter II**). Irrespective of origin, populations increased aboveground biomass and leaf N after a nutrient pulse, while the strength of this response differed among populations of *A. millefolium* and *B. hordeaceus* but not of *P. lanceolata*. In **Chapter II**, regrowth ability after a clipping treatment differed among populations of *A. millefolium* for total biomass and among populations of *P. lanceolata* for reproductive biomass, but populations of *B. hordeaceus* did not differ among each other. In fact, the amount of regrown biomass of clipped plants was mostly smaller but some populations achieved to regrow more biomass than their unclipped counterparts did. As such, a homeostatic response to tolerate aboveground damage was shown to differ widely among populations. Concluding, these experiments corroborate earlier findings of intraspecific population differentiation of plastic traits in a land-use context (Warwick and Briggs 1979; Carman and Briske 1985; Bergelson and Crawley 1992; Damhoureyeh and Hartnett 2002). However, also the lack of population differentiation in plastic responses, as found for *P. lanceolata* in response to fertilization and for *B. hordeaceus* in response to clipping in my experiments, has been shown in earlier studies (Rotundo and Aguiar 2007; Suzuki 2008; Gáspár *et al.* 2020). As indicated in **Chapter I,** and **II** this could be caused by strong gene flow among populations counteracting selection.

I also showed that the response of leaf chlorophyll content to a nutrient pulse differed among populations of *B. hordeaceus* and *P. lanceolata* at day 6 and day 10 after fertilization, respectively. However, the differences among populations ceased again 10 and 15 days after fertilization, respectively, demonstrating the importance of continuous measurements and defining leaf chlorophyll content as a process trait (cf. Volaire *et al.* 2020).

Besides focusing on opportunistic responses in **Chapter I** of my thesis, I could also investigate homeostatic responses to limited nutrient availability in the control group of *B. hordeaceus* and *P. lanceolata*. The ability to maintain photosynthesis at a constant level over a range of nutrient availabilities might be crucial for plant fitness or even survival. Interestingly, the homeostatic responses of conserving leaf chlorophyll in the control group visually differed among populations. Additionally, I detected a trade-off between the opportunistic response of increasing leaf chlorophyll content after fertilization, used as a proxy for nutrient acquisition, and the homeostatic response of maintaining leaf chlorophyll content under limited nutrient conditions, used as a proxy for nutrient conservation. This suggests that populations that respond opportunistically to fertilization suffer more under nutrient scarcity. This relates very well to known theory on plant resource use strategies (Wright *et al.* 2004; Reich 2014; Díaz *et al.* 2016) arguing that plants cannot be good at both, nutrient acquisition and conservation, but evolved towards either of the two strategies.

Association of plasticity with mean land-use intensity

In a second step in both **Chapter I**, and **II** I wanted to know if the strength of phenotypic plasticity was associated with land-use intensity, as was shown in some other studies on plasticity in a land-use context (Davies and Snaydon 1974; Carman and Briske 1985; Oesterheld and McNaughton 1988). For this purpose, I calculated a plasticity index as the log response ratio between treated (i.e. fertilized or clipped) and control plants and correlated this to several measures of land-use intensity. Overall, I found limited evidence for the association of the strength of plasticity and mean land-use intensity in both **Chapter I,** and **II**. In **Chapter I** of my thesis, I found that populations of *A. millefolium* exhibited lower plasticity to fertilization in terms of biomass and leaf N under higher mowing frequencies. Additionally, I detected a negative relationship between plasticity in biomass of *A. millefolium* and fertilization intensity. As fertilization intensity and mowing intensity are highly correlated processes in my study system (Völler *et al.* 2017; Vogt *et al.* 2019), I could not disentangle their effects on the evolution of plasticity in *A. millefolium*. However, mowing is known to be the land-use process with the strongest selective potential, as shown for several species within the Biodiversity Exploratories (Völler *et al.* 2013, 2017; Gossner *et al.* 2016), and might therefore also drive the evolution of phenotypic plasticity. In contrast to *A. millefolium*, plasticity of biomass and nitrogen related traits of both *B. hordeaceus* and *P. lanceolata* showed no association with mean intensity of land-use. Similar results emerged in **Chapter II**, where none of the species showed relationships between plasticity and mean land-use intensity.

Overall, there was little evidence that land-use intensity selected for phenotypic plasticity. However, the few negative relationships between plasticity and land-use intensity discovered in this thesis suggest that high fertilization and mowing intensity rather homogenize environmental conditions (Gossner *et al.* 2016) instead of creating heterogeneous environments, and therefore support the homogenization hypothesis.

Association of plasticity with temporal variation in land use In both **Chapter I**, and **II**, I investigated if temporal variation in land-use intensity, resulting from farmers changing their management practices from year to year and thereby creating additional heterogeneity, could have selected for increased plasticity. When relating the plasticity indices from both experiments to temporal variation in land-use intensity, I found little evidence for my expectation that plasticity should increase under more temporally variable grassland management. In **Chapter I**, the trade-off in nutrient acquisition vs. nutrient conservation in *P. lanceolata* showed a negative association with temporal variation in grazing intensity. In **Chapter II**, I detected a negative association of plasticity in reproductive biomass of *P. lanceolata* with temporal variation in mowing intensity, i.e. lower plasticity with increasing changes of mowing intensity among years. However, plasticity in total biomass of all three species did not relate to temporal variation in land use. The negative associations between plasticity and temporal variation in land use seemed surprising at first. However, several theoretical studies highlight the importance of the predictability of environmental heterogeneity for the evolution of plasticity (Scheiner 1993; Stuefer 1996; Alpert and Simms

2002; Lande 2009; Reed *et al.* 2010) and changing management practices among years reduces the reliability of management. Therefore, it might be plausible to find higher plasticity in more constantly managed grasslands and lower plasticity in grasslands that experience variable land-use activities among years.

The role of NSCs in regrowth ability of *P. lanceolata*

In **Chapter III** of my thesis, I investigated intraspecific differences in the storage of non-structural carbohydrates and their role in the regrowth ability of *P. lanceolata*. After only a few weeks of growth populations of *P. lanceolata* differed markedly in the amount of NSCs that are stored in its taproot. Relating this variation in NSC storage to land-use intensity, I found that mean mowing intensity as well as temporal variation in mowing intensity both decreased the amount of NSCs stored. This finding was contrary to what I expected, i.e. that higher levels of NSCs would be more advantageous under higher mowing intensities to repeatedly facilitate regrowth. In contrast, it could be possible that plants under high mowing intensities do not invest as much into storage but rather continuously invest in aboveground structures. Additionally, the time between two mowing events might be too short for a plant to assimilate and store enough NSCs to facilitate regrowth after the next mowing event. As such, storage of NSCs might not be the optimal strategy for plants under high mowing intensities and higher levels of NSCs would be expected under lower mowing intensities. The negative association between NSCs and temporal variation in mowing intensities is comparable to my discovery of decreasing plasticity of reproductive biomass of *P. lanceolata* under a more variable mowing regime (**Chapter II**). As mentioned above, the predictability of changes in environmental conditions such as caused by mowing might be crucial for the evolution of plastic responses as well as of increased storage to facilitate regrowth. Under a more variable mowing regime, it might thus be a better strategy to not rely on storage but invest in aboveground growth whenever possible.

It could be hypothesized that the regrowth ability of reproductive biomass of *P. lanceolata* depends on the level of NSCs before the removal of aboveground biomass, but I did not find such a relationship. The role of initial levels of NSCs before aboveground biomass removal for regrowth ability is not resolved yet, as some studies found associations (Danckwerts 1993; Turner *et al.* 2006; Lee *et al.* 2008) whereas others did not (Ryle and Powell 1975; Richards and Caldwell 1985). An explanation for a lack of a relationship could be that NSCs are only mobilized and facilitate regrowth for a short period of time after aboveground biomass removal (Visser *et al.* 1997; Morvan-Bertrand *et al.* 1999; Castrillón-Arbeláez *et al.* 2012) and that associations of initial levels of NSCs with regrowth ability fade over time (Morvan-Bertrand *et al.* 1999). Thus, it could be that initial levels of NSCs in my experiment related to regrowth ability shortly after the clipping treatment. However, as I harvested biomass 16 weeks after clipping, this association might have disappeared again. This resembles the fading trade-off between nutrient acquisition and conservation and highlights the importance of studying not only patterns but depending on the trait also processes (Volaire *et al.* 2020).

Conclusions and Outlook

With this thesis, I contributed to the scarce literature on the evolution of intraspecific phenotypic plasticity in grassland plants in response to land use. Earlier studies mostly compared only a few contrasting environments (e.g. grazed vs. non-grazed), whereas I used an extensive gradient of land-use intensity. Additionally, previous studies also remained limited in their spatial extent as well as in their level of replication. By using many populations from across Germany along a land-use gradient, I was able to extract a reliable signal of land-use intensity, more precisely mowing, grazing and fertilization intensity, on the evolution of phenotypic plasticity and thereby advanced our understanding of plastic responses to land use.

Overall, I found rather little evidence that populations of *A. millefolium*, *B. hordeaceus* and *P. lanceolata*, three common grassland plants in my study system, evolved plastic responses along a land-use gradient. However, the cases where I found associations between land use and plasticity suggested that lower land-use intensity selected for increased plastic responses. This highlights that low and intermediate land-use intensities might represent more fluctuating environments than high land-use intensities, such that increased plasticity would evolve under lower land-use intensity. As such, this is weak evidence for the homogenization hypothesis mentioned earlier.

I was surprised to find associations between the strength of plasticity and land-use intensity in only so few cases, since populations clearly differed in their response to the treatments for quite a few traits. Although population differentiation in plastic responses could result from differing selection pressures, i.e. land-use intensity, among grasslands, they could also result from genetic drift or from genetic linkage to other traits (Schlichting 1986). The fact that I only compared grasslands that are subject to at least some land use might explain the missing associations between phenotypic plasticity and land-use intensity. It could well be that plasticity is stronger in grassland populations that are managed compared to grasslands that do not experience recurring disturbances. However, as grasslands need at least some management to prevent bush encroachment (Milberg 1995) this seems impractical.

Besides the effect of land-use intensity on temporal heterogeneity in environmental conditions, land use also influences spatial patterns such as differences in number of species or species composition, microclimatic conditions, or topography. Especially grazing creates a large variety of micro-environments within a grassland, whereas mowing homogenizes conditions over space. One could thus use data on spectral heterogeneity on different scales as well as vegetation observations to investigate the association between spatial heterogeneity and phenotypic plasticity. Additionally, the variation in responses among populations could also have been driven by other environmental variables, such as soil fertility, precipitation or climate. I would thus suggest that future studies should analyze other environmental variables that potentially drive the evolution of phenotypic plasticity.

An additional question that should be answered while studying the evolution of phenotypic plasticity, is whether the observed response is adaptive or not. By definition, the term phenotypic plasticity does not imply an adaptive advantage of plastic responses but merely describes active and/or passive responses to an environmental stimulus. Plasticity is only considered adaptive if plastic responses increase fitness across environments. To evaluate the adaptiveness of a plastic response, it would be necessary to measure fitness or at least fitness-related traits of individuals in all environments (e.g. control and treated individuals). Unfortunately, during my common garden experiments, reproductive output was low in all three species, such that I could not evaluate the adaptiveness of the observed plastic responses.

In this thesis, I investigated the evolution of intraspecific phenotypic plasticity in a land-use context. I found ample variation in phenotypic plasticity, meaning that grassland populations differ among each other in plastic responses. This type of intraspecific variation is an important part of biodiversity and thus worthy of protection. Firstly, because it is a necessary asset for adaptation to future environmental changes (e.g. land-use change) which benefits populations and species and, through supporting ecosystem services, even our livelihood. Secondly, because biodiversity has intrinsic value and should therefore be protected and preserved.

Bibliography

Adler P, Raff D, Lauenroth W (2001) The effect of grazing on the spatial heterogeneity of vegetation. *Oecologia* **128**:465–479.

Aerts R, Chapin FS (1999) The Mineral Nutrition of Wild Plants Revisited: A Re-evaluation of Processes and Patterns. In AH Fitter and DG Raffaelli (eds). *Advances in Ecological Research*. Academic Press, 1–67.

Agrawal AA (2000) Overcompensation of plants in response to herbivory and the by-product benefits of mutualism. *Trends in Plant Science* **5**:309–313.

Ainouche ML, Bayer RJ, Gourret J-P, Defontaine A, Misset M-T (1999) The allotetraploid invasive weed *Bromus hordeaceus* L. (Poaceae): Genetic diversity, origin and molecular evolution. *Folia Geobotanica* **34**:405–419.

Allan E, Bossdorf O, Dormann CF, *et al.* (2014) Interannual variation in land-use intensity enhances grassland multidiversity. *Proceedings of the National Academy of Sciences* **111**:308–313.

Alpert P, Simms EL (2002) The relative advantages of plasticity and fixity in different environments: when is it good for a plant to adjust? *Evolutionary Ecology* **16**:285–297.

Auld JR, Agrawal AA, Relyea RA (2010) Re-evaluating the costs and limits of adaptive phenotypic plasticity. *Proceedings of the Royal Society B: Biological Sciences* **277**:503–511.

Balaguer L, Martínez-Ferri E, Valladares F, *et al.* (2001) Population Divergence in the Plasticity of the Response of *Quercus coccifera* to the Light Environment. *Functional Ecology* **15**:124–135.

Baptist F, Secher-Fromell H, Viard-Cretat F, *et al.* (2013) Carbohydrate and nitrogen stores in *Festuca paniculata* under mowing explain dominance in subalpine grasslands. *Plant Biology* **15**:395–404.

Barton KE (2013) Ontogenetic patterns in the mechanisms of tolerance to herbivory in Plantago. *Annals of Botany* **112**:711–720.

Bates D, Mächler M, Bolker B, Walker S (2015) Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software* **67**:1–48.

Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B (Methodological)* **57**:289–300.

Benot M-L, Morvan-Bertrand A, Mony C, *et al.* (2019) Grazing intensity modulates carbohydrate storage pattern in five grass species from temperate grasslands. *Acta Oecologica* **95**:108–115.

Benton TG, Vickery JA, Wilson JD (2003) Farmland biodiversity: is habitat heterogeneity the key? *Trends in Ecology & Evolution* **18**:182–188.

Berg H, Becker U, Matthies D (2005) Phenotypic plasticity in *Carlina vulgaris*: effects of geographical origin, population size, and population isolation. *Oecologia* **143**:220–231.

Bergelson J, Crawley MJ (1992) Herbivory and *Ipomopsis aggregata*: The Disadvantages of Being Eaten. *The American Naturalist* **139**:870–882.

Bloor J, Pottier J (2014) Grazing and spatial heterogeneity: Implications for grassland structure and function. In: P. Mariotte & P. Kardol (eds.) Grassland Biodiversity and Conservation in a Changing World, pp. 135– 162. Nova Science, New York, NY, US.

Blüthgen N, Dormann CF, Prati D, *et al.* (2012) A quantitative index of land-use intensity in grasslands: Integrating mowing, grazing and fertilization. *Basic and Applied Ecology* **13**:207–220.

Boot RGA, Mensink M (1990) Size and morphology of root systems of perennial grasses from contrasting habitats as affected by nitrogen supply. *Plant and Soil* **129**:291–299.

Bourdôt GW (1984) Regeneration of yarrow (*Achillea millefolium* L.) rhizome fragments of different length from various depths in the soil. *Weed Research* **24**:421–429.

Bourdôt GW, Field RJ (1988) Review on ecology and control of *Achillea millefolium* L. (yarrow) on arable land in New Zealand. *New Zealand Journal of Experimental Agriculture* **16**:99–108.

Bowsher AW, Miller BJ, Donovan LA (2016) Evolutionary divergences in root system morphology, allocation, and nitrogen uptake in species from high- versus low-fertility soils. *Functional Plant Biology* **43**:129–140.

Bradshaw AD (1965) Evolutionary Significance of Phenotypic Plasticity in Plants. In EW Caspari and JM Thoday (eds). *Advances in Genetics*. Academic Press, 115–155.

Briggs D (2009) *Plant Microevolution and Conservation in Human-influenced Ecosystems*. Cambridge University Press.

CABI (2020), *Achillea millefolium* [original text by Jeanine Vélez-Gavilán]. In: Invasive Species Compendium. Wallingford, UK: CAB International. www.cabi.org/isc.

CABI (2020), *Bromus hordeaceus* [original text by John Peter Thompson]. In: Invasive Species Compendium. Wallingford, UK: CAB International. www.cabi.org/isc.

CABI (2020), *Plantago lanceolata*. In: Invasive Species Compendium. Wallingford, UK: CAB International. www.cabi.org/isc.

Carman JG, Briske DD (1985) Morphologic and allozymic variation between long-term grazed and non-grazed populations of the bunchgrass *Schizachyrium scoparium* var. frequens. *Oecologia* **66**:332–337.

Carpenter LT, Pezeshki SR, Shields FD (2008) Responses of nonstructural carbohydrates to shoot removal and soil moisture treatments in *Salix nigra*. *Trees* **22**:737–748.

Carroll SP, Hendry AP, Reznick DN, Fox CW (2007) Evolution on ecological time-scales. *Functional Ecology* **21**:387–393.

Castrillón-Arbeláez PA, Martínez-Gallardo N, Arnaut HA, Tiessen A, Délano-Frier JP (2012) Metabolic and enzymatic changes associated with carbon mobilization, utilization and replenishment triggered in grain amaranth (*Amaranthus cruentus*) in response to partial defoliation by mechanical injury or insect herbivory. *BMC Plant Biology* **12**:163.

Cavers PB, Bassett IJ, Crompton CW (1980) The Biology of Canadian Weeds: 47. *Plantago lanceolata* L. *Canadian Journal of Plant Science* **60**:1269–1282.

Chapin FS (1980) The Mineral Nutrition of Wild Plants. *Annual Review of Ecology and Systematics* **11**:233–260.

Chapin FS, Schulze E, Mooney HA (1990) The Ecology and Economics of Storage in Plants. *Annual Review of Ecology and Systematics* **21**:423–447.

Clayton, W.D., Vorontsova, M.S., Harman, K.T. and Williamson, H. (2006 onwards). GrassBase - The Online World Grass Flora. http://www.kew.org/data/grasses-db.html. [accessed 13 October 2006; 15:30 GMT]

Crick JC, Grime JP (1987) Morphological Plasticity and Mineral Nutrient Capture in Two Herbaceous Species of Contrasted Ecology. *New Phytologist* **107**:403–414.

Damhoureyeh SA, Hartnett DC (2002) Variation in grazing tolerance among three tallgrass prairie plant species. *American Journal of Botany* **89**:1634–1643.

Danckwerts JE (1993) Reserve Carbon and Photosynthesis: Their Role in Regrowth of *Themeda Triandra*, a Widely Distributed Subtropical Graminaceous Species. *Functional Ecology* **7**:634–641.

Danckwerts JE, Gordon AJ (1987) Long-term Partitioning, Storage and Re-mobilization of 14C Assimilated by *Lolium perenne* (cv. Melle). *Annals of Botany* **59**:55–66.

Davies A (1965) Carbohydrate levels and regrowth in perennial rye-grass. *The Journal of Agricultural Science* **65**:213–221.

Davies MS, Snaydon RW (1974) Physiological Differences Among Populations of *Anthoxanthum odoratum* L. Collected from the Park Grass Experiment, Rothamsted. III. Response to Phosphate. *The Journal of Applied Ecology* **11**:699.

Davis MB, Shaw RG (2001) Range Shifts and Adaptive Responses to Quaternary Climate Change. *Science* **292**:673–679.

Díaz S, Hodgson JG, Thompson K, *et al.* (2004) The plant traits that drive ecosystems: Evidence from three continents. *Journal of Vegetation Science* **15**:295–304.

Díaz S, Kattge J, Cornelissen JHC, *et al.* (2016) The global spectrum of plant form and function. *Nature* **529**:167–171.

Díaz S, Lavorel S, McIntyre S, *et al.* (2007) Plant trait responses to grazing – a global synthesis. *Global Change Biology* **13**:313–341.

Díaz S, Settele J, Brondízio E, *et al.* (2019) Summary for policymakers of the global assessment report on biodiversity and ecosystem services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services.

Dietze MC, Sala A, Carbone MS, *et al.* (2014) Nonstructural Carbon in Woody Plants. *Annual Review of Plant Biology* **65**:667–687.

Donaghy DJ, Fulkerson WJ (1997) The importance of water-soluble carbohydrate reserves on regrowth and root growth of *Lolium perenne* (L.). *Grass and Forage Science* **52**:401–407.

Donaghy, Fulkerson (1998) Priority for allocation of water-soluble carbohydrate reserves during regrowth of *Lolium perenne*. *Grass and Forage Science* **53**:211–218.

Dong M, During HJ, Werger MJA (1996) Morphological responses to nutrient availability in four clonal herbs. *Vegetatio* **123**:183–192.

Donohue K, Pyle EH, Messiqua D, Heschel MS, Schmitt J (2001) Adaptive Divergence in Plasticity in Natural Populations of *Impatiens Capensis* and Its Consequences for Performance in Novel Habitats. *Evolution* **55**:692–702.

Dostál P, Fischer M, Prati D (2016) Phenotypic plasticity is a negative, though weak, predictor of the commonness of 105 grassland species. *Global Ecology and Biogeography* **25**:464–474.

Drenovsky RE, Khasanova A, James JJ (2012) Trait convergence and plasticity among native and invasive species in resource-poor environments. *American Journal of Botany* **99**:629–639.

Erbilgin N, Galvez DA, Zhang B, Najar A (2014) Resource availability and repeated defoliation mediate compensatory growth in trembling aspen (*Populus tremuloides*) seedlings. *PeerJ* **2**:e491.

Fischer M, Bossdorf O, Gockel S, *et al.* (2010) Implementing large-scale and long-term functional biodiversity research: The Biodiversity Exploratories. *Basic and Applied Ecology* **11**:473–485.

Foley JA, DeFries R, Asner GP, *et al.* (2005) Global Consequences of Land Use. *Science* **309**:570–574.

Foley JA, Ramankutty N, Brauman KA, *et al.* (2011) Solutions for a cultivated planet. *Nature* **478**:337–342.

Foster L (1988) Herbs in Pastures. Development Research in Britain, 1850–1984. *Biological Agriculture & Horticulture* **5**:97–133.

Fransen B, Blijjenberg J, Kroon H de (1999) Root morphological and physiological plasticity of perennial grass species and the exploitation of spatial and temporal heterogeneous nutrient patches. *Plant and Soil* **211**:179–189.

Fransen B, Kroon H de, Berendse F (1998) Root morphological plasticity and nutrient acquisition of perennial grass species from habitats of different nutrient availability. *Oecologia* **115**:351–358.

Fritz C, Lamers LPM, Riaz M, Berg LJL van den, Elzenga TJTM (2014) Sphagnum Mosses - Masters of Efficient N-Uptake while Avoiding Intoxication. *PLOS ONE* **9**:e79991.

Fuller RJ, Gough SJ (1999) Changes in sheep numbers in Britain: implications for bird populations. *Biological Conservation* **91**:73–89.

Gagliardi S, Martin AR, Filho E de MV, Rapidel B, Isaac ME (2015) Intraspecific leaf economic trait variation partially explains coffee performance across agroforestry management regimes. *Agriculture, Ecosystems & Environment* **200**:151–160.

Gáspár B, Bossdorf O, Durka W (2019) Structure, stability and ecological significance of natural epigenetic variation: a large-scale survey in *Plantago lanceolata*. *New Phytologist* **221**:1585–1596.

Gáspár B, Bossdorf O, Parepa M (2020) Intraspecific variation in land use-related functional traits in *Plantago lanceolata*. *bioRxiv* 2020.02.28.967521.

Gianoli E (2004) Plasticity of Traits and Correlations in Two Populations of *Convolvulus arvensis* (Convolvulaceae) Differing in Environmental Heterogeneity. *International Journal of Plant Sciences* **165**:825–832.

Gianoli E, González-Teuber M (2005) Environmental Heterogeneity and Population Differentiation in Plasticity to Drought in *Convolvulus Chilensis* (Convolvulaceae). *Evolutionary Ecology* **19**:603–613.

Gianoli E, Valladares F (2012) Studying phenotypic plasticity: the advantages of a broad approach. *Biological Journal of the Linnean Society* **105**:1–7.

Gibson SI (2005) Control of plant development and gene expression by sugar signaling. *Current Opinion in Plant Biology* **8**:93–102.

Gibson DJ, Urban J, Baer SG (2011) Mowing and fertilizer effects on seedling establishment in a successional old field. *Journal of Plant Ecology* **4**:157–168.

Gossner MM, Lewinsohn TM, Kahl T, *et al.* (2016) Land-use intensification causes multitrophic homogenization of grassland communities. *Nature* **540**:266–269.

Grainger TN, Turkington R (2013) Long-term nutrient enrichment differentially affects investment in sexual reproduction in four boreal forest understory species. *Plant Ecology* **214**:1017–1026.

Grassein F, Till-Bottraud I, Lavorel S (2010) Plant resource-use strategies: the importance of phenotypic plasticity in response to a productivity gradient for two subalpine species. *Annals of Botany* **106**:637–645.

Greub LJ, Wedin WF (1971) Leaf Area, Dry-Matter Production, and Carbohydrate Reserve Levels of Birdsfoot Trefoil as Influenced by Cutting Height 1. *Crop Science* **11**:734–738.

Grime JP, Mackey JML (2002) The role of plasticity in resource capture by plants. *Evolutionary Ecology* **16**:299–307.

Harrison XA, Donaldson L, Correa-Cano ME, *et al.* (2018) A brief introduction to mixed effects modelling and multi-model inference in ecology. *PeerJ* **6**.

Hartfield M, Bataillon T, Glémin S (2017) The Evolutionary Interplay between Adaptation and Self-Fertilization. *Trends in Genetics* **33**:420–431.

Hartmann H, Ziegler W, Trumbore S (2013) Lethal drought leads to reduction in nonstructural carbohydrates in Norway spruce tree roots but not in the canopy. *Functional Ecology* **27**:413–427.

Heilmeier H, Schulze E-D, Whale DM (1986) Carbon and nitrogen partitioning in the biennial monocarp *Arctium tomentosum* Mill. *Oecologia* **70**:466–474.

Hoch G (2015) Carbon Reserves as Indicators for Carbon Limitation in Trees. In U Lüttge and W Beyschlag (eds). *Progress in Botany: Vol. 76*. Cham: Springer International Publishing, 321–346.

Hoch G, Richter A, Körner Ch (2003) Non-structural carbon compounds in temperate forest trees. *Plant, Cell & Environment* **26**:1067–1081.

Hodge A (2004) The plastic plant: root responses to heterogeneous supplies of nutrients. *New Phytologist* **162**:9–24.

Hodgkinson KC (1969) The Utilization of Root Organic Compounds During the Regeneration of Lucerne. *Australian Journal of Biological Sciences* **22**:1113–1124.

Hoffmann AA, Sgrò CM (2011) Climate change and evolutionary adaptation. *Nature* **470**:479–485.

Hogg EH, Lieffers VJ (1991) Seasonal changes in shoot regrowth potential in *Calamagrostis canadensis*. *Oecologia* **85**:596–602.

Hoogesteger J, Karlsson PS (1992) Effects of Defoliation on Radial Stem Growth and Photosynthesis in the Mountain Birch (*Betula pubescens ssp. tortuosa*). *Functional Ecology* **6**:317–323.

Horibata S, Hasegawa SF, Kudo G (2007) Cost of Reproduction in a Spring Ephemeral Species, *Adonis ramosa* (Ranunculaceae): Carbon Budget for Seed Production. *Annals of Botany* **100**:565–571.

Huhta A-P, Hellström K, Rautio P, Tuomi J (2003) Grazing tolerance of *Gentianella amarella* and other monocarpic herbs: why is tolerance highest at low damage levels? *Plant Ecology* **166**:49–61.

Hume DE (1991) Effect of Cutting on Production and Tillering in Prairie Grass (*Bromus willdenowii* Kunth) Compared With Two Ryegrass (Loliunt) Species. 1. Vegetative Plants. *Annals of Botany* **67**:533–541.

Hutchings MJ, Kroon H de (1994) Foraging in Plants: the Role of Morphological Plasticity in Resource Acquisition. In M Begon and AH Fitter (eds). *Advances in Ecological Research*. Academic Press, 159–238.

Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services I (2019) *Summary for policymakers of the global assessment report on biodiversity and ecosystem services*. Zenodo.

Isaac ME, Martin AR, Melo Virginio Filho E de, Rapidel B, Roupsard O, Van den Meersche K (2017) Intraspecific Trait Variation and Coordination: Root and Leaf Economics Spectra in Coffee across Environmental Gradients. *Frontiers in Plant Science* **8**.

Janeček Š, Klimešová J (2014) Carbohydrate storage in meadow plants and its depletion after disturbance: do roots and stem-derived organs differ in their roles? *Oecologia* **175**:51–61.

Janeček Š, Lanta V, Klimešová J, Doležal J (2011) Effect of abandonment and plant classification on carbohydrate reserves of meadow plants. *Plant Biology* **13**:243–251.

Jong GD (1999) Unpredictable selection in a structured population leads to local genetic differentiation in evolved reaction norms. *Journal of Evolutionary Biology* **12**:839–851.

Jump AS, Peñuelas J (2005) Running to stand still: adaptation and the response of plants to rapid climate change. *Ecology Letters* **8**:1010–1020.

Kirschbaum A, Bossdorf O, Scheepens JF (2021) Variation in regrowth ability in relation to land use intensity in three common grasslands herbs. *Journal of Plant Ecology* **14**:438-450.

Krasensky J, Jonak C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *Journal of Experimental Botany* **63**:1593–1608.

Kreyling J, Schweiger AH, Bahn M, *et al.* (2018) To replicate, or not to replicate – that is the question: how to tackle nonlinear responses in ecological experiments. *Ecology Letters* **21**:1629–1638.

Kuiper D (1984) Genetic differentiation and phenotypic plasticity in Plantago species. Ph.D. Thesis, University of Groningen

Kuiper PJC, Bos M (eds) (1992) *Plantago: A Multidisciplinary Study*. Berlin Heidelberg: Springer-Verlag.

Kuznetsova A, Brockhoff PB, Christensen RHB (2017) lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software* **82**:1–26.

Lande R (2009) Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. *Journal of Evolutionary Biology* **22**:1435–1446.

Landhäusser SM, Chow PS, Dickman LT, *et al.* (2018) Standardized protocols and procedures can precisely and accurately quantify non-structural carbohydrates. M Mencuccini (ed). *Tree Physiology* **38**:1764–1778.

Landhäusser SM, Lieffers VJ (2002) Leaf area renewal, root retention and carbohydrate reserves in a clonal tree species following above-ground disturbance. *Journal of Ecology* **90**:658–665.

Latzel V, Janeček Š, Doležal J, Klimešová J, Bossdorf O (2014) Adaptive transgenerational plasticity in the perennial *Plantago lanceolata*. *Oikos* **123**:41–46.

Latzel V, Janeček Š, Hájek T, Klimešová J (2014) Biomass and Stored Carbohydrate Compensation after Above-Ground Biomass Removal in a Perennial Herb: Does Environmental Productivity Play a Role? *Folia Geobotanica* **49**:17–29.

Lázaro-Nogal A, Matesanz S, Godoy A, Pérez-Trautman F, Gianoli E, Valladares F (2015) Environmental heterogeneity leads to higher plasticity in dry-edge populations of a semi-arid Chilean shrub: insights into climate change responses. W Cornwell (ed). *Journal of Ecology* **103**:338–350.

Lee JM, Donaghy DJ, Roche JR (2008) Effect of Defoliation Severity on Regrowth and Nutritive Value of Perennial Ryegrass Dominant Swards. *Agronomy Journal* **100**:308–314.

Lee JM, Minnee EMK, Clark CEF (2015) Patterns in non-structural carbohydrate and nitrogen reserves in chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.) during regrowth in summer. *Crop and Pasture Science* **66**:1071–1078.

Lee JM, Sathish P, Donaghy DJ, Roche JR (2010) Plants Modify Biological Processes to Ensure Survival following Carbon Depletion: A *Lolium perenne* Model. F Harmon (ed). *PLoS ONE* **5**:e12306.

Leiss KA, Müller-Schärer H (2001) Adaptation of *Senecio vulgaris* (Asteraceae) to ruderal and agricultural habitats. *American Journal of Botany* **88**:1593–1599.

Lennartsson T, Nilsson P, Tuomi J (1998) Induction of Overcompensation in the Field Gentian, Gentianella Campestris. *Ecology* **79**:1061–1072.

Lennartsson T, Tuomi J, Nilsson P (1997) Evidence for an Evolutionary History of Overcompensation in the Grassland Biennial *Gentianella Campestris* (Gentianaceae). *The American Naturalist* **149**:1147–1155.

Li H, Hoch G, Körner C (2002) Source/sink removal affects mobile carbohydrates in *Pinus cembra* at the Swiss treeline. *Trees* **16**:331–337.

Louault F, Pillar VD, Aufrère J, Garnier E, Soussana J-F (2005) Plant traits and functional types in response to reduced disturbance in a semi-natural grassland. *Journal of Vegetation Science* **16**:151–160.

Martin AR, Rapidel B, Roupsard O, *et al.* (2017) Intraspecific trait variation across multiple scales: the leaf economics spectrum in coffee. *Functional Ecology* **31**:604–612.

Martínez-Vilalta J, Sala A, Asensio D, *et al.* (2016) Dynamics of non-structural carbohydrates in terrestrial plants: a global synthesis. *Ecological Monographs* **86**:495–516.

Matesanz S, Gianoli E, Valladares F (2010) Global change and the evolution of phenotypic plasticity in plants: Global change and plasticity. *Annals of the New York Academy of Sciences* **1206**:35–55.

Matesanz S, Horgan-Kobelski T, Sultan SE (2012) Phenotypic Plasticity and Population Differentiation in an Ongoing Species Invasion. *PLOS ONE* **7**:e44955.

Matson PA (1997) Agricultural Intensification and Ecosystem Properties. *Science* **277**:504–509.

McNaughton SJ (1979) Grazing as an Optimization Process: Grass-Ungulate Relationships in the Serengeti. *The American Naturalist* **113**:691–703.

McNaughton SJ (1983) Compensatory Plant Growth as a Response to Herbivory. *Oikos* **40**:329–336.

Milberg P (1995) Soil Seed Bank after Eighteen Years of Succession from Grassland to Forest. *Oikos* **72**:3.

Mitchell-Olds T, Willis JH, Goldstein DB (2007) Which evolutionary processes influence natural genetic variation for phenotypic traits? *Nature Reviews Genetics* **8**:845–856.

Morgan JM (1984) Osmoregulation and Water Stress in Higher Plants. *Annual Review of Plant Physiology* **35**:299–319.

Morvan-Bertrand A, Boucaud J, Prud'homme M-P (1999) Influence of initial levels of carbohydrates, fructans, nitrogen, and soluble proteins on regrowth of *Lolium perenne* L. cv. Bravo following defoliation. *Journal of Experimental Botany* **50**:1817–1826.

Morvan-Bertrand A, Pavis N, Boucaud J, Prud'Homme M-P (1999) Partitioning of reserve and newly assimilated carbon in roots and leaf tissues of *Lolium perenne* during regrowth after defoliation: assessment by 13C steady-state labelling and carbohydrate analysis. *Plant, Cell & Environment* **22**:1097–1108.

Munns R, Tester M (2008) Mechanisms of Salinity Tolerance. *Annual Review of Plant Biology* **59**:651–681.

Niinemets Ü (2015) Is there a species spectrum within the world-wide leaf economics spectrum? Major variations in leaf functional traits in the Mediterranean sclerophyll *Quercus ilex*. *New Phytologist* **205**:79–96.

Oesterheld M, McNaughton SJ (1988) Intraspecific variation in the response of *Themeda triandra* to defoliation: the effect of time of recovery and growth rates on compensatory growth. *Oecologia* **77**:181–186.

Olden JD, LeRoy Poff N, Douglas MR, Douglas ME, Fausch KD (2004) Ecological and evolutionary consequences of biotic homogenization. *Trends in Ecology & Evolution* **19**:18–24.

Osone Y, Tateno M (2005) Applicability and Limitations of Optimal Biomass Allocation Models: A Test of Two Species from Fertile and Infertile Habitats. *Annals of Botany* **95**:1211–1220.

Ostrowski A, Lorenzen K, Petzold E, Schindler S (2020) *Land use intensity index (LUI) calculation tool of the Biodiversity Exploratories project for grassland survey data from three different regions in Germany since 2006, BEXIS 2 module*. Zenodo.

Palacio S, Hernández R, Maestro-Martínez M, Camarero JJ (2012) Fast replenishment of initial carbon stores after defoliation by the pine processionary moth and its relationship to the re-growth ability of trees. *Trees* **26**:1627–1640.

Pigliucci M (2001) *Phenotypic Plasticity: Beyond Nature and Nurture*. JHU Press.

Pigliucci M (2005) Evolution of phenotypic plasticity: where are we going now? *Trends in Ecology & Evolution* **20**:481–486.

Pigliucci M (2006) Phenotypic plasticity and evolution by genetic assimilation. *Journal of Experimental Biology* **209**:2362–2367.

Pigliucci M, Murren CJ (2003) Perspective: Genetic Assimilation and a Possible Evolutionary Paradox: Can Macroevolution Sometimes Be so Fast as to Pass Us By? *Evolution* **57**:1455–1464.

R Core Team (2019) *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.*

Ramankutty N, Foley JA (1999) Estimating historical changes in global land cover: Croplands from 1700 to 1992. *Global Biogeochemical Cycles* **13**:997–1027.

Reed TE, Waples RS, Schindler DE, Hard JJ, Kinnison MT (2010) Phenotypic plasticity and population viability: the importance of environmental predictability. *Proceedings of the Royal Society B: Biological Sciences* **277**:3391–3400.

Reich PB (2014) The world-wide 'fast–slow' plant economics spectrum: a traits manifesto. *Journal of Ecology* **102**:275–301.

Reich PB, Walters MB, Ellsworth DS (1997) From tropics to tundra: Global convergence in plant functioning. *PNAS* **94**:13730–13734.

Reisch C, Poschlod P (2009) Land use affects flowering time: seasonal and genetic differentiation in the grassland plant *Scabiosa columbaria*. *Evolutionary Ecology* **23**:753–764.

Reisch C, Poschlod P (2011) Morphology and phenology of *Scabiosa columbaria* from mown and grazed habitats – Results of a simulation experiment. *Flora - Morphology, Distribution, Functional Ecology of Plants* **206**:887–891.

Rejmánek M (2000) Invasive plants: approaches and predictions. *Austral Ecology* **25**:497–506.

Relyea RA, Morin AEPJ (2002) Costs of Phenotypic Plasticity. *The American Naturalist* **159**:272–282.

Richards CL, Bossdorf O, Muth NZ, Gurevitch J, Pigliucci M (2006) Jack of all trades, master of some? On the role of phenotypic plasticity in plant invasions. *Ecology Letters* **9**:981–993.

Richards JH, Caldwell MM (1985) Soluble Carbohydrates, Concurrent Photosynthesis and Efficiency in Regrowth Following Defoliation: A Field Study with Agropyron Species. *The Journal of Applied Ecology* **22**:907.

Robinson BW, Dukas R (1999) The Influence of Phenotypic Modifications on Evolution: The Baldwin Effect and Modern Perspectives. *Oikos* **85**:582–589.

Rosenthal JP, Kotanen PM (1994) Terrestrial plant tolerance to herbivory. *Trends in Ecology & Evolution* **9**:145–148.

Rotundo JL, Aguiar MR (2007) Herbivory resistance traits in populations of *Poa ligularis* subjected to historically different sheep grazing pressure in Patagonia. *Plant Ecology* **194**:121–133.

Roumet C, Birouste M, Picon-Cochard C, *et al.* (2016) Root structure–function relationships in 74 species: evidence of a root economics spectrum related to carbon economy. *New Phytologist* **210**:815–826.

Ryle GJA, Powell CE (1975) Defoliation and Regrowth in the Graminaceous Plant: The Role of Current Assimilate. *Annals of Botany* **39**:297–310.

Sartori K, Vasseur F, Violle C, *et al.* (2019) Leaf economics and slow-fast adaptation across the geographic range of *Arabidopsis thaliana*. *Scientific Reports* **9**:10758.

Schädel C, Richter A, Blöchl A, Hoch G (2010) Hemicellulose concentration and composition in plant cell walls under extreme carbon source–sink imbalances. *Physiologia Plantarum* **139**:241–255.

Scheiner SM (1993) Genetics and Evolution of Phenotypic Plasticity. *Annual Review of Ecology and Systematics* **24**:35–68.

Scheiner SM (2013) The genetics of phenotypic plasticity. XII. Temporal and spatial heterogeneity. *Ecology and Evolution* **3**:4596–4609.

Schlichting CD (1986) The Evolution of Phenotypic Plasticityin Plants. *Annual Review of Ecology and Systematics* 29.

Schlichting CD, Levin DA (1986) Phenotypic plasticity: an evolving plant character. *Biological Journal of the Linnean Society* **29**:37–47.

Schlichting CD, Levin DA (1990) Phenotypic plasticity in Phlox. III. Variation among natural populations of *P. drummondii*. *Journal of Evolutionary Biology* **3**:411–428.

Schnyder H, Visser R de (1999) Fluxes of Reserve-Derived and Currently Assimilated Carbon and Nitrogen in Perennial Ryegrass Recovering from Defoliation. The Regrowing Tiller and Its Component Functionally Distinct Zones. *Plant Physiology* **119**:1423–1436.

Shipley B, Lechowicz MJ, Wright I, Reich PB (2006) Fundamental Trade-Offs Generating the Worldwide Leaf Economics Spectrum. *Ecology* **87**:535–541.

Silvertown J, Poulton P, Johnston E, Edwards G, Heard M, Biss PM (2006) The Park Grass Experiment 1856–2006: its contribution to ecology. *Journal of Ecology* **94**:801–814.

Simms EL (2000) Defining tolerance as a norm of reaction. *Evolutionary Ecology* **14**:563–570.

Smith AM, Stitt M (2007) Coordination of carbon supply and plant growth. *Plant, Cell & Environment* **30**:1126–1149.

Snaydon RW, Davies MS (1972) Rapid Population Differentiation in a Mosaic Environment. II. Morphological Variation in *Anthoxanthum odoratum*. *Evolution* **26**:390–405.

Socher SA, Prati D, Boch S, *et al.* (2013) Interacting effects of fertilization, mowing and grazing on plant species diversity of 1500 grasslands in Germany differ between regions. *Basic and Applied Ecology* **14**:126–136.

Steffen W, Broadgate W, Deutsch L, Gaffney O, Ludwig C (2015) The trajectory of the Anthropocene: The Great Acceleration. *The Anthropocene Review* **2**:81–98.

Strauss SY, Agrawal AA (1999) The ecology and evolution of plant tolerance to herbivory. *Trends in Ecology & Evolution* **14**:179–185.

Stuefer JF (1996) Potential and limitations of current concepts regarding the response of clonal plants to environmental heterogeneity. *Vegetatio* **127**:55–70.

Sultan SE (1987) Evolutionary Implications of Phenotypic Plasticity in Plants. In MK Hecht, B Wallace, and GT Prance (eds). *Evolutionary Biology: Volume 21*. Boston, MA: Springer US, 127–178.

Sultan SE (1995) Phenotypic plasticity and plant adaptation. *Acta Botanica Neerlandica* **44**:363–383.

Sultan SE (2000) Phenotypic plasticity for plant development, function and life history. *Trends in Plant Science* **5**:537–542.

Sultan SE (2003) Phenotypic plasticity in plants: a case study in ecological development. *Evolution and Development* **5**:25–33.

Sultan SE, Spencer HG (2002) Metapopulation Structure Favors Plasticity over Local Adaptation. *The American Naturalist* **160**:271–283.

Sultan SE, Wilczek AM, Bell DL, Hand G (1998) Physiological response to complex environments in annual Polygonum species of contrasting ecological breadth. *Oecologia* **115**:564–578.

Suzuki RO (2008) Dwarf morphology of the annual plant *Persicaria longiseta* as a local adaptation to a grazed habitat, Nara Park, Japan. *Plant Species Biology* **23**:174–182.

Tiffin P (2000) Mechanisms of tolerance to herbivore damage: what do we know? *Evolutionary Ecology* **14**:523–536.

Tilman D (2001) Forecasting Agriculturally Driven Global Environmental Change. *Science* **292**:281–284.

Tufto J (2000) The Evolution of Plasticity and Nonplastic Spatial and Temporal Adaptations in the Presence of Imperfect Environmental Cues. *The American Naturalist* **156**:121–130.

Turner LR, Donaghy DJ, Lane PA, Rawnsley RP (2006) Effect of defoliation management, based on leaf stage, on perennial ryegrass (*Lolium perenne* L.), prairie grass (*Bromus willdenowii* Kunth.) and cocksfoot (*Dactylis glomerata* L.) under dryland conditions. 1. Regrowth, tillering and water-soluble carbohydrate concentration. *Grass and Forage Science* **61**:164–174.

Valladares F, Gianoli E, Gómez JM (2007) Ecological limits to plant phenotypic plasticity. *New Phytologist* **176**:749–763.

Van de Vijver CADM, Boot RGA, Poorter H, Lambers H (1993) Phenotypic plasticity in response to nitrate supply of an inherently fast-growing species from a fertile habitat and an inherently slow-growing species from an infertile habitat. *Oecologia* **96**:548–554.

Van Kleunen M, Fischer M (2001) Adaptive Evolution of Plastic Foraging Responses in a Clonal Plant. *Ecology* **82**:3309–3319.

Van Kleunen M, Fischer M (2005) Constraints on the evolution of adaptive phenotypic plasticity in plants: Research review. *New Phytologist* **166**:49–60.

Van Tienderen PHV, Toorn J van der (1991) Genetic Differentiation Between Populations of *Plantago Lanceolata* . I. Local Adaptation in Three Contrasting Habitats. *The Journal of Ecology* **79**:27.

Vergeer P, Berg LLJVD, Bulling MT, Ashmore MR, Kunin WE (2008) Geographical variation in the response to nitrogen deposition in *Arabidopsis lyrata petraea*. *New Phytologist* **179**:129–141.

Via S, Gomulkiewicz R, De Jong G, Scheiner SM, Schlichting CD, Van Tienderen PH (1995) Adaptive phenotypic plasticity: consensus and controversy. *Trends in Ecology & Evolution* **10**:212–217.

Visser RD, Vianden H, Schnyder H (1997) Kinetics and relative significance of remobilized and current C and N incorporation in leaf and root growth zones of *Lolium perenne* after defoliation: assessment by 13C and 15N steady-state labelling. *Plant, Cell & Environment* **20**:37–46.

Vitousek PM, Naylor R, Crews T, *et al.* (2009) Nutrient Imbalances in Agricultural Development. *Science* **324**:1519–1520.

Vogt J, Klaus V, Both S, *et al.* (2019) Eleven years' data of grassland management in Germany. *Biodiversity Data Journal* **7**:e36387.

Volaire F, Gleason SM, Delzon S (2020) What do you mean "functional" in ecology? Patterns versus processes. *Ecology and Evolution* **10**:11875–11885.

Volis S, Mendlinger S, Ward D (2002) Differentiation in populations of *Hordeum spontaneum* Koch along a gradient of environmental productivity and predictability: plasticity in response to water and nutrient stress. *Biological Journal of the Linnean Society* **75**:301–312.

Völler E, Auge H, Bossdorf O, Prati D (2013) Land use causes genetic differentiation of life-history traits in *Bromus hordeaceus*. *Global Change Biology* **19**:892–899.

Völler E, Bossdorf O, Prati D, Auge H (2017) Evolutionary responses to land use in eight common grassland plants. H Jacquemyn (ed). *Journal of Ecology* **105**:1290–1297.

Wang D, Du J, Zhang B, Ba L, Hodgkinson KC (2017) Grazing Intensity and Phenotypic Plasticity in the Clonal Grass *Leymus chinensis*. *Rangeland Ecology & Management* **70**:740–747.

Warwick SI, Black L (1982) The Biology of Canadian Weeds: 52. *Achillea millefolium* L. S.L. *Canadian Journal of Plant Science* **62**:163–182.

Warwick SI, Briggs D (1979) The Genecology of Lawn Weeds. III. Cultivation Experiments with *Achillea millefolium* L., *Bellis perennis* L., *Plantago lanceolata* L., *Plantago major* L. and *Prunella vulgaris* L. Collected from Lawns and Contrasting Grassland Habitats. *The New Phytologist* **83**:509–536.

Waters CN, Zalasiewicz J, Summerhayes C, *et al.* (2016) The Anthropocene is functionally and stratigraphically distinct from the Holocene. *Science* **351**.

Wedlich KV, Vergeer P, Ashmore MR, Berg LLJ van den (2016) The importance of considering origin in effects of nitrogen deposition on plant performance and competitive ability. *Plant and Soil* **401**:307–318.

Weemstra M, Mommer L, Visser EJW, *et al.* (2016) Towards a multidimensional root trait framework: a tree root review. *New Phytologist* **211**:1159–1169.

Weinig C (2000) Plasticity Versus Canalization: Population Differences in the Timing of Shade-Avoidance Responses. *Evolution* **54**:441–451.

West-Eberhard MJ (1989) Phenotypic Plasticity and the Origins of Diversity. *Annual Review of Ecology and Systematics* **20**:249–278.

West-Eberhard MJ (2003) *Developmental Plasticity and Evolution*. Oxford University Press.

Wijesinghe DK, John EA, Beurskens S, Hutchings MJ (2001) Root system size and precision in nutrient foraging: responses to spatial pattern of nutrient supply in six herbaceous species. *Journal of Ecology* **89**:972–983.

Wise MJ, Abrahamson WG (2007) Effects of Resource Availability on Tolerance of Herbivory: A Review and Assessment of Three Opposing Models. *The American Naturalist* **169**:443–454.

Wolff K, Van Delden W (1987) Genetic analysis of ecological relevant morphological variability in *Plantago lanceolata* L. I Population characteristics. *Heredity* **58**:183–192.

Wright IJ, Reich PB, Westoby M, *et al.* (2004) The worldwide leaf economics spectrum. *Nature* **428**:821–827.