Evolutionary consequences of land use – Epigenetic and phenotypic variation in *Plantago lanceolata*

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Declaration of author contributions

The thesis entitled “Evolutionary consequences of land use – Epigenetic and phenotypic variation in *Plantago lanceolata*” is based on the work I did during my PhD at University of Tübingen, supervised by Prof. Dr. Oliver Bossdorf and by Dr. Walter Durka (Centre for Environmental Research – UFZ; Halle, Germany; German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Germany), in collaboration with Dr. Madalin Parepa. In this thesis, Chapters II–IV 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 II

OB and WD planned and designed the research; BG conducted fieldwork, performed experiments and laboratory work; and all authors analysed the data and wrote the manuscript.

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Chapter III

OB and WD planned and designed the research; BG conducted fieldwork and performed experiments; BG and OB analysed the data and wrote the manuscript with input from WD.

Chapter IV

BG and MP designed the experiment with the help of OB. BG and MP performed the experiment. BG and MP analysed the data. BG and OB wrote the manuscript with input from MP.

Summary

Human activity is resulting in extreme changes to Earth's biotic and abiotic systems, and in 2019, this information is finally reaching the general public, too. While the most famous phenomenon is climate warming due to the burning of fossil fuels, land use intensification – including the expansion of managed area and the more intense use of existing agricultural fields – is the main cause of the loss of biological diversity. Land plants as primary producers are at the foundation of terrestrial ecosystems (ironically also at the foundation of our own agriculture), and understanding how their evolution has been and will be shaped by anthropogenic activity is crucial for human societies.

Past evolutionary events and potential for future evolution can be inferred from two types of variation within species: the differentiation between populations, and the diversity within populations (being the raw material for evolution), respectively. Within-species variation has also profound effects on the production, stability and resilience of plant populations, and these effects also propagate across whole plant communities and ecosystems. Studies of plant phenotypic and genetic variation in human-managed ecosystems, especially mown and grazed grasslands, have established that differentiation between close but differently managed areas can take place within a couple of generations, showing the prevalence of rapid evolution. Most recently, research on epigenetic variation – primarily focusing on DNA methylation – has gained momentum, as it became clear that it can also result in phenotypic differences and that natural plant populations are variable on this level, too. However, comprehensive studies on the effect of land use on intraspecific variation are lacking, as well as on the epigenetic variation in wild populations of non-model plants. Furthermore, studies on land use and intraspecific variation often focussed on agriculturally interesting traits, with less attention to ecologically relevant plant traits. In this thesis, I aimed to narrow these gaps by asking: (i) How much intraspecific genetic, epigenetic, and phenotypic variation is there within (diversity) and among (differentiation) wild plant populations? (ii) How is genetic, epigenetic, and phenotypic variation related to each other? (iii) What is their relationship to environmental factors, especially to land use intensity? (iv) Are there trade-offs or positive correlations between functional traits relevant in grasslands?

I worked with *Plantago lanceolata*, a very common grassland plant, and took advantage of the network of standardised study plots with detailed land-use information across Germany, that is hosted by the Biodiversity Exploratories research platform. In Chapter II, I focused on the analysis of epigenetic variation in field- and common-garden-collected material, and related it to genetic and phenotypic variation, environmental variables and land use intensity. In Chapter III, I analysed the phenotypic data from the same material in more detail, and explored heritable variation in the measured traits, and the relationship of their population-level means and diversities to land use intensity. In Chapter IV, I zoomed in on three traits important in grasslands; quantifying the competitive ability, response to nutrient pulses and clipping/grazing tolerance of *P. lanceolata* via an inventive greenhouse experiment, and examined whether there is significant variation in these traits, as well as their relationships to land use, and between each other.

I found that: (i) There is substantial epigenetic, genetic and phenotypic variation in *P. lanceolata*, mostly as within-population diversity, but still showing significant differentiation among populations. (ii) There was no detectable relationship between the three levels of intraspecific variation I studied. (iii) Increasing mowing intensity decreases epigenetic and phenotypic diversity, and the opposite is true for their relationship to grazing intensity; while genetic variation was unrelated to land use. (iv) Nutrient pulse response and clipping tolerance in *P. lanceolata* are negatively correlated, probably representing a physiological trade-off, while a positive correlation between competitive ability and clipping tolerance was most likely present because they confer benefits in the same environments. Altogether, these results show that rapid evolution associated with land use has taken place in *P. lanceolata*, even if this species is wind-pollinated and strictly outcrossing, which results in high levels of gene-flow and much unstructured variation. This also means that there is plenty of “raw material” for future evolution in this system, as well as the potential for finding stronger associations between these three levels of intraspecific variation and environmental variables in other species. Extending this kind of research, with more high-resolution genomic and epigenomic methods would certainly contribute to our understanding of rapid evolution in human-influenced ecosystems.

Zusammenfassung

Menschliches Handeln führen zu extremen Veränderungen in den biotischen und abiotischen Systemen der Erde, in 2019 erreicht dieses Wissen endlich die breite Öffentlichkeit. Globale Erwärmung infolge der Nutzung fossiler Brennstoffe ist das Bekannteste unter diesen Phänomenen, während jedoch Landnutzungs-intensivierung – sowohl die Verbreitung landwirtschaftlicher Gebiete als auch die intensivere Nutzung der bereits existierenden Agrarflächen – die Hauptursache für den Verlust biologischer Diversität ist. Pflanzen sind als primäre Produzenten die Basis terrestrischer Ökosysteme (ironischerweise auch die unserer Landwirtschaft). Daher ist das Verständnis dafür, wie ihre Evolution von menschlichem Handeln beeinflusst wurde und wird, entscheidend für menschliche Gesellschaften.

Informationen über vergangene evolutionäre Ereignisse und Potenzial für zukünftige Evolution können aus zwei Kategorien von innerartlicher Variation erschlossen werden: erstens aus der Differenzierung zwischen Populationen und zweitens aus der Diversität innerhalb von Populationen (das Rohmaterial für Evolution). Die Produktivität, Stabilität und Resilienz von Pflanzenpopulationen wird auch stark von innerartlicher Variation beeinflusst: deren Effekte können Einfluss auf ganze Pflanzengemeinschaften und Ökosysteme haben. Studien über phäno-typische und genetische Variation von Pflanzen in menschlich genutzten Öko-systemen, vor allem gemähter und beweideter Grünlandflächen, haben bewiesen, dass eine Differenzierung zwischen nah beieinander liegenden aber unterschiedlich genutzten Landflächen innerhalb von ein paar Generationen stattfinden kann. Dies belegt die Prävalenz rapider Evolution. In jüngster Zeit hat die Erforschung epigenetischer Variation – vor allem der DNA-Methylierung – an Dynamik gewonnen, da deutlich wurde, dass sie ebenfalls zu phänotypischen Unterschieden führen kann und dass natürliche Pflanzenpopulationen auch auf dieser Ebene variabel sind. Umfassende Studien über den Einfluss der Landnutzung auf die intraspezifische Variation fehlen jedoch ebenso wie Studien über die epigenetische Variation innerhalb wilder Populationen von Nicht-Modellpflanzen. Darüber hinaus konzentrierten sich die Studien zur Landnutzung und intraspezifischen Variation oft auf Merkmale die für die landwirtschaftliche Nutzung wichtig sind, während ökologisch relevante Pflanzenmerkmale weniger berücksichtigt wurden. In dieser Arbeit habe ich versucht, diese Wissenlücken durch folgende Fragen zu verringern:

(i) Wie viel intraspezifische genetische, epigenetische und phänotypische Variation gibt es innerhalb (Diversität) und zwischen (Differenzierung) wilde Pflanzenpopulationen? (ii) Wie hängen genetische, epigenetische und phänotypische Variation zusammen? (iii) In welchem Verhältnis stehen sie zu Umweltfaktoren, insbesondere zur Intensität der Landnutzung? (iv) Gibt es Trade-Offs oder positive Korrelationen zwischen für Grünlandflächen relevante funktionale Merkmale?

Ich arbeitete mit *Plantago lanceolata*, einer weit verbreiteten Grünlandpflanze, und nutzte das deutschlandweite Netzwerk standardisierter Untersuchungsflächen mit detaillierten Landnutzungsinformation, das von der Forschungsplattform der Biodiversitäts-Exploratorien betrieben wird. In Kapitel II konzentrierte ich mich auf die Analyse der epigenetischen Variation von Feld- und „Common Garden"-Pflanzen und setze diese in Bezug zu genetischer und phänotypischer Variation, Umwelt-variablen und Landnutzungsintensität. In Kapitel III analysierte ich die phäno-typischen Daten aus dem gleichen Material detaillierter und untersuchte die erbliche Variation der gemessenen Merkmale auf Populationsebene, und wie deren Mittelwerte und Diversität mit der Landnutzungsintensität zusammenhängen. In Kapitel IV habe ich drei für Grünlandpflanzen wichtige Merkmale untersucht; Konkurrenzfähigkeit, Reaktion auf Nährstoffimpulse und Mäh-/Beweidungstoleranz. Diese wurden für *P. lanceolata* mit Hilfe eines Gewächshaus-Experimentes quantifiziert und ich habe untersucht, ob es signifikante Variation in diesen Merkmalen gibt, sowie wie sie untereinander und mit Landnutzung in Beziehung stehen.

Meine Ergebnisse zeigen: (i) Es gibt erhebliche epigenetische, genetische und phänotypische Variation bei *P. lanceolata*, meist in Form von Diversität innerhalb der Population, mit signifikanter Differenzierung zwischen den Populationen. (ii) Es gab keinen erkennbaren Zusammenhang zwischen den drei Ebenen der intra-spezifischen Variation, die ich untersucht habe. (iii) Zunehmende Mähintensität verringert die epigenetische und phänotypische Vielfalt, das Gegenteil gilt für Beweidungsintensität, während es zwischen Landnutzung und genetischer Variation keine Beziehung gibt. (iv) Die Nährstoffimpulsantwort und die Mähtoleranz bei *P. lanceolata* sind negativ korreliert und stellen höchstwahrscheinlich einen physiologischen Trade-Off dar, während eine positive Korrelation zwischen

Konkurrenzfähigkeit und Mähtoleranz bestand, vermutlich weil diese Vorteile in den gleichen Umgebungen bieten. Insgesamt zeigen diese Ergebnisse, dass bei *P. lanceolata* eine rasche Evolution im Zusammenhang mit Landnutzung stattgefunden hat, und das obwohl diese Art windbestäubt und streng auskreuzend ist, was zu einem hohen Grad an Genfluss und viel unstrukturierter Variation führt. Das bedeutet auch, dass es in diesem System viel "Rohmaterial" für zukünftige Evolution gibt und dass potenziell stärkere Assoziationen zwischen diesen drei Ebenen der intraspezifischen Variation und den Umweltvariablen in anderen Arten gefunden werden können. Die Erweiterung dieser Art von Forschung durch hochauflösendere genomische und epigenomische Methoden würde sicherlich zu unserem Verständnis der raschen Evolution in vom Menschen beeinflussten Ökosystemen beitragen.

Chapter I
General Introduction

1. Intraspecific variation in plants

Differences between individuals of a given species exist in all possible examined characters. The heritable fraction of this within-species variation is the raw material of evolution, however, before the confirmation of DNA's role in heredity in the 1950's and the advent of molecular techniques in the 1960's, the only measurable manifestation of intraspecific variation was the phenotype. Until the pioneering work of Turesson (1922), scholars mostly thought that local variants of the same plant species were induced by their specific habitat, and struggled with separating the plastic and heritable portions of intraspecific phenotypic variation (Briggs & Walters, 2016). In the early 20th century, the modern synthesis of evolution reconciled the continuous distribution of the observed phenotypes and the discrete units of inheritance, giving way to population genetics and quantitative genetics, using initially only the phenotypes to infer underlying evolutionary processes (selection, mutation, drift, and gene flow). So there is a strong history of plant evolutionary biologists investigating within-species variation, and documenting evolution in action – often in grasslands and/or agricultural settings (Stapledon, 1928; Snaydon, 1970; Warwick & Briggs, 1978; Wolff & Van Delden, 1987; Van Tienderen & van der Toorn, 1991; Núñez-Farfán & Schlichting, 2001; Vergeer et al., 2003).

Since the 1960's, molecular genetic – and more recently, genomic – technologies have allowed to quantify genetic variation directly. Most of the variation captured by genetic markers is neutral (ie. resulting from mutation, drift, or gene flow), and is only weakly correlated with adaptive variation (ie. resulting from selection). It is therefore often argued that neutral genetic variation is not a good predictor of the adaptive potential of a population (Reed & Frankham, 2001; Mittell *et al.*, 2015). However, it is known that part of the harboured genetic variation only results in heritable phenotypic variation under unusual circumstances (cryptic genetic variation, CGV; Paaby & Rockman, 2014), and that adaptation from standing (ie. pre-existing) genetic variation is faster than from newly arisen mutations (Barrett &

Schluter, 2008). It has also been pointed out by Colautti *et al.* (2012) that even if there has been a careful mapping of quantitative traits to the well-characterised genome of the model plant, *Arabidopsis thaliana* in the greenhouse, few of these loci would be detected in natural conditions, while many new candidates would appear that have not been found under controlled conditions.

Besides the central role of intraspecific variation in evolution, its effects ripple through many levels of organisation, including, populations, communities and ecosystems (Hughes *et al.*, 2008). Higher genotypic diversity improves production (Crutsinger *et al.*, 2006) and the stability thereof (Prieto *et al.*, 2015), and confers higher resistance to invasions (Crutsinger *et al.*, 2008) and diseases (Mundt, 2002), as well as resilience in the face of climatic extremes (Reusch *et al.*, 2005; Ehlers *et al.*, 2008). Intraspecific variation also affects higher levels; it reduces species diversity decline (Booth & Grime, 2003), and modulates community assembly (Jung *et al.*, 2010) and multitrophic interactions (Reusch *et al.*, 2005; Crutsinger *et al.*, 2006). In the field of functional and trait-based community ecology (Violle *et al.*, 2007), a recent shift away from the perspective that within-species variation should be negligible compared to between-species variation (McGill *et al.*, 2006) has now lead to intense inquiry of “intraspecific trait variability” (ITV; Violle *et al.*, 2012). Individual studies of this line of research showed that mean values of single species may hide substantial functional variation in the community (Albert *et al.*, 2010 b; Siefert *et al.*, 2015), and reduce observed effects by lumping together distinct species-specific responses (Kichenin *et al.*, 2013). In some cases, the effect of intraspecific variation has even been found to be stronger than variation on the species level (Cook-Patton *et al.*, 2011; Des Roches *et al.*, 2018). Ecosystem-level effects of intraspecific variation, such as decomposition (Madritch *et al.*, 2006) and nutrient cycling (Hughes & Stachowicz, 2004) have been documented. Apart from all the different aspects of the importance of intraspecific variation listed above (and discussed elsewhere; see Mimura *et al.*, 2017), it is also a level of biodiversity with intrinsic value (Ghilarov, 2000).

2. Epigenetic variation

In the past decade, epigenetic variation, as it can result in phenotypic variation in the absence of underlying genetic variation (Cubas *et al.*, 1999; Cortijo *et al.*, 2014), has also started to take its place in the evolutionary biology of natural populations (Bossdorf *et al.*, 2008; Richards *et al.*, 2017). Epigenetic mechanisms modulate gene expression and contribute to genome structure and integrity through a concerted interplay of chemical modifications of the DNA (eg. methylation of cytosines) and chromatin (e.g. histone methylation and acetylation, spatial arrangement), as well as small RNAs. These processes play a central role in development and tissue differentiation, and the information they hold is heritable between mitotically multiplying cell populations, and can even be transmitted over generations on the organismal level (Weigel & Colot, 2012). Epigenetic variation has recently gotten much attention, as its heritability is combined with the environmental inducibility, and so has the potential of accelerating evolution (Gutzat & Mittelsten Scheid, 2012).

In plants, presumably because of their sessile lifestyle and the value of the mother plant's environmental information, epigenetic regulation is more complex than in animals. DNA methylation, the most well-studied epigenetic mechanism occurs in three sequence contexts in plants (CG, CHG and CHH, where H = A, G, or T), with distinct responsible molecular pathways, and different functions (Zhang *et al.*, 2018a). Some important types of methylated regions are i) promoters, mostly resulting in repressed transcription; ii) gene bodies in the CG context, associated with slow-evolving housekeeping genes; and iii) silenced transposable elements (TEs) with heavy methylation in all three contexts (Bewick & Schmitz, 2017; Zhang *et al.*, 2018a). Most of our knowledge on plant epigenetics comes from the model plant *Arabidopsis thaliana*, and the crop species *Oryza sativa* and *Zea mays* (rice and maize). There are several lines of evidence from epigenetic recombinant inbred lines (Reinders *et al.*, 2009; epiRILs; Johannes *et al.*, 2009) from *Arabidopsis* that show that variation mainly in DNA methylation (and very little in the DNA sequence), can result in substantial heritable phenotypic variation (Zhang *et al.*, 2013, 2018b; Cortijo *et al.*, 2014; Kooke *et al.*, 2015), that eventually can be subjected to selection. A positive diversity effect, similar to that known for species and genetic diversity, has

been also demonstrated on the productivity and stability of experimental epiRIL populations (Latzel *et al.*, 2013). However, *A. thaliana* has an exceptionally small genome (~135 Mb) with low global methylation (5% vs 10–40% in other species; Alonso *et al.*, 2015) and unusual organisation: most of the TEs and no genes are at the centromeres, and that is where most of the methylation is (Seymour *et al.*, 2014). The genomes of rice and maize for instance are substantially larger (430 Mb and 2.4 Gb), and have very different epigenomic organisations, with TEs and DNA methylation much more distributed across their chromosomes (Li *et al.*, 2012, 2014).

In order to assess the generality and realism of the mechanisms and patterns explored in the *A. thaliana* and crop systems, it is important to investigate epigenetic variation also in natural populations of other species. This venture has started about a decade ago (Bossdorf *et al.*, 2008), and has yielded much knowledge and even more questions about natural epigenetic variation, especially in non-model species. Briefly, there is much evidence that epigenetic variation exists in natural populations, however, in order to disentangle its genetic dependence, its environmentally induced versus unaffected, transient versus heritable fractions, and its phenotypic effects, the examination of many populations, with a comprehensive experimental design, and possibly extensive genomic resources is needed. For a more exhaustive discussion of this topic see Chapter 2 and Richards *et al.* (2017).

Altogether, there is accumulating evidence from model species in laboratory conditions (Cortijo *et al.*, 2014; Kooke *et al.*, 2015; Zhang *et al.*, 2018b) and proof-of-principle studies from natural populations of wild species (Herrera & Bazaga, 2010; Lira-Medeiros *et al.*, 2010; Richards *et al.*, 2012; Medrano *et al.*, 2014; Schulz *et al.*, 2014, Groot *et al.*, 2018) that epigenetic variation can also harbour substantial evolutionary potential, however, to assess its true importance in realistic settings, further research is needed. Understanding the evolutionary potential of plant populations is especially crutial today, as it is expected to play an important role in the context of global environmental change.

3. Plants in the Anthropocene

We live in the Anthropocene, a geological epoch defined by human activity (Crutzen, 2002; Lewis & Maslin, 2015). Human activity has changed the climate and major biogeochemical cycles of the Earth (Steffen *et al.*, 2007). We have connected every corner of the world, and bring organisms with or without intention to new habitats (van Kleunen *et al.*, 2015). We also halved plant biomass on Earth over the past 5000 years (Smil, 2013), and exchanged most vertebrate biomass to domesticated animals; today, humans and their domesticated livestock make up for 96% of all mammal biomass, and 70% of bird biomass comes in some form of poultry (Bar-On *et al.*, 2018). In 2005, 40% of land were croplands and pastures (Foley *et al.*, 2005), most of which is sprayed with fertiliser, herbicides, pesticides, and antibiotics. Also, 35% of the total CO_2 emissions between 1850 and 1990 came directly from land use (Houghton, 1999). The list could go on why humans earned the title of the “world’s greatest evolutionary force” (Palumbi, 2001).

All of these anthropogenic factors act on plant populations indirectly through direct agents of selection, which can be grouped into abiotic (climatic or soil conditions) and biotic factors (competitors, herbivores, pollinators, seed dispersers, symbionts or pathogens; Cheplick, 2015), and these direct effects have been studied extensively (see Linhart and Grant, 1996 and Cheplick, 2015 for a comprehensive listing of studies). The consequences of anthropogenic global change have been also documented widely, however mostly on higher organisational levels, such as shifts in distribution ranges, phenology, community composition, and extinctions (Parmesan, 2006), and changes on the intraspecific level are understudied. While there are some studies for example on evolutionary consequences of global change (Franks *et al.*, 2014, and other papers in the same special issue) or invasion (Bossdorf *et al.*, 2005; Colautti & Lau, 2015), more research has to be conducted on the relationship of intraspecific variation and land use.

4. Land use and plant evolution

Land use is globally the strongest factor contributing to biodiversity loss (Díaz *et al.*, 2019). Its detrimental effects exceed those of climate change, nitrogen deposition,

biotic exchange, and the rise of atmospheric CO_2 level in seven out of twelve biomes examined by Sala *et al.* (2000). Besides increasing the areas under management, leading to habitat loss and reduction, the intensification of already existing land use also threatens wildlife (Sala *et al.*, 2000; Foley *et al.*, 2005; Newbold *et al.*, 2015). Contemporary land use practices alter global biogeochemical cycles of nitrogen, phosphorus, carbon, and water via fertilisation, irrigation and tillage leading to soil erosion, thereby changing trophic networks and the structure of ecological communities (Foley *et al.*, 2005, and references therein).

Grasslands cover about a quarter of land area, represent ⅔ of agricultural land (da Silveira Pontes *et al.*, 2015), and have been historically important in agriculture, specifically for animal husbandry, so the ecophysiology and adaptations of plants in these ecosystems have been extensively studied (Lemaire *et al.*, 2000; Briggs, 2009). This has mostly been done by analysing functional traits, traits that influence the fitness – growth, survival, and reproduction – of a plant (Violle *et al.*, 2007). It turns out that many of these traits co-vary, while others do not, and this leads to the concept of trait syndromes, one of the most well-known of which is the leaf economic spectrum (Wright *et al.*, 2004), distributing plants along an axis between the exploitative (high specific leaf area, leaf N content, photosynthetic rate, and short life span) and conservative (opposing trait combination) strategies. Another prominent theory describing plant functional strategies places species in the so-called CSR triangle (Grime *et al.*, 2014). C-strategist species (competitors) grow tall with a high light interception, S-strategists (stress tolerators) are well adapted to stressful environments, have a deep root system and are long-lived, while R-strategists (ruderals) complete their life cycle and disperse rapidly. A third framework that emerged from the study of functional traits is the leaf-height-seed (LHS) strategy scheme (Westoby, 1998), that uses three traits: leaf mass per area, height, and seed mass to classify a plant's ecological strategy.

The frameworks outlined above have been applied to study the effect of the main filtering forces in grasslands (i.e. nutrient availability, defoliation, and competition) on plant species. The trait syndromes associated with a gradient of nutrient availability follow the leaf economic spectrum (i.e. exploitative strategy at

nutrient rich sites). Interestingly, high growth rate is also associated with frequent defoliation, while conservative strategies adaptive at nutrient-poor habitats are also increasing resistance to herbivory (da Silveira Pontes *et al.*, 2015 and references therein). Strategies adaptive for high competition could range from structural and physiological components of shade tolerance and shade avoidance (Niinemets, 2010), to belowground processes such as nutrient exploitation (Tilman, 1985) or microbial interactions (Bever *et al.*, 2010); a comprehensive enumeration is however beyond the scope of this introduction. Most of this research has, however, focused on the number of species, and despite the "return of the variance" (Violle *et al.*, 2012), more information is needed on other levels of biological diversity, such as intraspecific genetic, epigenetic or phenotypic variation.

It is known from a number of studies that mowing and grazing in general, can select for prostrate growth forms (Warwick & Briggs, 1979; Aarssen & Turkington, 1985; Rotundo & Aguiar, 2008; Suzuki, 2008), that fertilisation increases light competition, and selects for more vigorous growth (Hautier *et al.*, 2009), and that different mowing times can lead to heritable changes in phenology (Zopfi, 1993; Völler *et al.*, 2013). However, almost all of the studies investigating the relationship of land use and intraspecific variation studies are based on a limited number of populations and restricted geographical range (see Briggs, 2009, chap. 8 for a comprehensive list of studies), so additional detailed research based on larger samples is necessary (as exemplified by Völler *et al.*, 2013, and 2017). The connection between epigenetic variation and land use has also not been explored so far, pointing out directions for further research.

5. Study species

Plantago lanceolata is a herbaceous perennial grassland plant species, originally native to Eurasia but now globally distributed. It is one of the most common grassland herbs across its distribution range, and so is a species with high ecological relevance (Sagar & Harper, 1964). Its leaves form a rosette and the flowers are born in compact inflorescences on long scapes. It is wind-pollinated and self-incompatible, diploid, and has a genome size of 1n ≈ 1.28 Gb. It mostly flowers readily in the first

year, and germinates after cold stratification (Pons, 1992), all of which characteristics make it a favourable species for experimental, ecological, and genetic studies. Other European species in the genus also have a similar biology, and a large research project between the late 1970's and early 1990's in the Netherlands – combining ecology, population genetics, physiology, soil sciences, and microbiology among others – produced over 200 academic papers that were summarised in the book 'Plantago: A Multidisciplinary Study' (Kuiper & Bos, 1992).

Much of the work in this project was carried out with *P. lanceolata*, and many of these studies focused on population genetic questions, even in relation to land use. These pieces of research identified among others: local adaptation (Van Tienderen & van der Toorn, 1991), substantially different life histories (van Groenendael, 1986), and population differentiation in 17 phenotypic traits (Wolff & Van Delden, 1987) in relation to land use. However, all of these studies were restricted to a handful of populations (two to four in the above mentioned cases), and thus the questions of generalisability and whether the differences were indeed due to land use remain open. Other earlier research on *P. lanceolata* found no heritable phenotypic variation in five phenotypic traits in relation to four different categories of land use (Warwick & Briggs, 1979).

6. Goals of my thesis

As outlined in this Introduction, intraspecific variation has a crucial role in plant eco-evolutionary dynamics, however, an important part of it – epigenetic variation – is lacking large-scale studies conducted on wild populations of non-model species. Moreover, whereas the effect of land-use intensification on biological diversity has been extensively studied, its consequences for intraspecific variation and rapid evolution are understudied.

In **Chapter II** of this thesis, I analysed the epigenetic, genetic, and phenotypic variation (diversity and differentiation) coming from *P. lanceolata* material that I collected in 60 wild populations across Germany. I followed a comprehensive experimental design proposed by (Bossdorf *et al.*, 2008), including the comparison of field-collected and common-garden-derived material that enables the discernment of

environmentally induced and stable epigenetic variation. I also examined the relationships between intraspecific epigenetic, genetic and phenotypic variation, and their association with three different components of land-use intensity (mowing, fertilisation, and grazing) in the managed grasslands of the original populations.

In **Chapter III**, I analysed the phenotypic traits measured in the common garden experiment in greater detail. The main inquiry in this chapter was whether there is substantial heritable phenotypic variation that could serve as raw material for evolution, and whether land use could be a consistent and strong enough selective force for adaptation to occur. I analysed the population-level means and diversities of nine different phenotypic traits associated with plant size, leaf economy, and reproduction, and related them to the mowing, fertilisation, and grazing intensities of the original field sites in order to answer these questions.

In **Chapter IV**, I conducted another greenhouse experiment, where I explored in detail three functional traits related to land use processes. In managed grasslands, plants are constantly exposed to competition, and regularly experience addition of nutrients (as fertiliser or livestock droppings), and removal of biomass, and accordingly, I quantified competitive ability as R^* (Tilman, 1985), response to a nutrient pulse, and clipping tolerance. I examined their relationship to mowing, fertilisation, and grazing intensity, as well as their interconnectedness – to find out whether there are trade-offs or positive genetic correlations between them.

References

Aarssen LW, Turkington R. 1985. Within-species diversity in natural populations of *Holcus lanatus*, *Lolium perenne* and *Trifolium repens* from four different-aged pastures. *The Journal of Ecology* 73: 869.

Albert CH, Thuiller W, Yoccoz NG, Soudant A, Boucher F, Saccone P, Lavorel S. 2010. Intraspecific functional variability: extent, structure and sources of variation. *The Journal of Ecology* 98: 604–613.

Alonso C, Pérez R, Bazaga P, Herrera CM. 2015. Global DNA cytosine methylation as an evolving trait: phylogenetic signal and correlated evolution with genome size in angiosperms. *Frontiers in Genetics* 6: 4.

Bar-On YM, Phillips R, Milo R. 2018. The biomass distribution on Earth. *Proceedings of the National Academy of Sciences of the United States of America* 115: 6506–6511.

Barrett RDH, Schluter D. 2008. Adaptation from standing genetic variation. *Trends in Ecology & Evolution* 23: 38–44.

Bever JD, Dickie I a., Facelli E, Facelli JM, Klironomos J, Moora M, Rillig MC, Stock WD, Tibbett M, Zobel M. 2010. Rooting theories of plant community ecology in microbial interactions. *Trends in Ecology & Evolution* 25: 468–478.

Bewick AJ, Schmitz RJ. 2017. Gene body DNA methylation in plants. *Current Opinion in Plant Biology* 36: 103–110.

Booth RE, Grime JP. 2003. Effects of genetic impoverishment on plant community diversity: Effects of genetic impoverishment. *The Journal of Ecology* 91: 721–730.

Bossdorf O, Auge H, Lafuma L, Rogers WE, Siemann E, Prati D. 2005. Phenotypic and genetic differentiation between native and introduced plant populations. *Oecologia* 144: 1–11.

Bossdorf O, Richards CL, Pigliucci M. 2008. Epigenetics for ecologists. *Ecology Letters* 11: 106–115.

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

Briggs D, Walters S. 2016. *Plant Variation and Evolution*. Cambridge University Press.

Cheplick GP. 2015. *Approaches to Plant Evolutionary Ecology*. Oxford University Press.

Colautti RI, Lau JA. 2015. Contemporary evolution during invasion: evidence for differentiation, natural selection, and local adaptation. *Molecular Ecology* 24: 1999–2017.

Colautti RI, Lee C-R, Mitchell-Olds T. 2012. Origin, fate, and architecture of ecologically relevant genetic variation. *Current Opinion in Plant Biology* 15: 199–204.

Cook-Patton SC, McArt SH, Parachnowitsch AL, Thaler JS, Agrawal AA. 2011. A direct comparison of the consequences of plant genotypic and species diversity on communities and ecosystem function. *Ecology* 92: 915–923.

Cortijo S, Wardenaar R, Colomé-Tatché M, Gilly A, Etcheverry M, Labadie K, Caillieux E, Hospital F, Aury J-M, Wincker P, *et al.* 2014. Mapping the epigenetic basis of complex traits. *Science* 343: 1145–1148.

Crutsinger GM, Collins MD, Fordyce JA, Gompert Z, Nice CC, Sanders NJ. 2006. Plant genotypic diversity predicts community structure and governs an ecosystem process. *Science* 313: 966–968.

Crutsinger GM, Souza L, Sanders NJ. 2008. Intraspecific diversity and dominant genotypes resist plant invasions. *Ecology Letters* 11: 16–23.

Crutzen PJ. 2002. Geology of mankind. *Nature* 415: 23.

Cubas P, Vincent C, Coen E. 1999. An epigenetic mutation responsible for natural variation in floral symmetry. *Nature*: 157–161.

Des Roches S, Post DM, Turley NE, Bailey JK, Hendry AP, Kinnison MT, Schweitzer JA, Palkovacs EP. 2018. The ecological importance of intraspecific variation. *Nature Ecology & Evolution* 2: 57–64.

Díaz S, Settele J, Brondízio E, Ngo H, Guèze M, Agard J, Arneth A, Balvanera P, Brauman K, Butchart S, *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.

Ehlers A, Worm B, Reusch TBH. 2008. Importance of genetic diversity in eelgrass *Zostera marina* for its resilience to global warming. *Marine Ecology Progress Series* 355: 1–7.

Foley JA, Defries R, Asner GP, Barford C, Bonan G, Carpenter SR, Chapin FS, Coe MT, Daily GC, Gibbs HK, *et al.* 2005. Global consequences of land use. *Science* 309: 570–574.

Franks SJ, Weber JJ, Aitken SN. 2014. Evolutionary and plastic responses to climate change in terrestrial plant populations. *Evolutionary Applications* 7: 123–139.

Ghilarov AM. 2000. Ecosystem functioning and intrinsic value of biodiversity. *Oikos* 90: 408–412.

Grime JP, Hodgson JG, Hunt R. 2014. *Comparative Plant Ecology: A Functional Approach to Common British Species*. Springer.

van Groenendael JM. 1986. Life history characteristics of two ecotypes of *Plantago lanceolata* L. *Acta Botanica Neerlandica* 35: 71–86.

Groot MP, Wagemaker N, Ouborg NJ, Verhoeven KJF, Vergeer P. 2018. Epigenetic population differentiation in field- and common garden-grown *Scabiosa columbaria* plants. *Ecology* 8: 3505–3517.

Gutzat R, Mittelsten Scheid O. 2012. Epigenetic responses to stress: triple defense? *Current Opinion in Plant Biology* 15: 568–573.

Hautier Y, Niklaus PA, Hector A. 2009. Competition for light causes plant biodiversity loss after eutrophication. *Science* 324: 636–638.

Houghton RA. 1999. The annual net flux of carbon to the atmosphere from changes in land use 1850–1990. *Tellus. Series B, Chemical and Physical Meteorology* 51: 298–313.

Hughes AR, Inouye BD, Johnson MTJ, Underwood N, Vellend M. 2008. Ecological consequences of genetic diversity. *Ecology Letters* 11: 609–623.

Hughes AR, Stachowicz JJ. 2004. Genetic diversity enhances the resistance of a seagrass ecosystem to disturbance. *Proceedings of the National Academy of Sciences of the United States of America* 101: 8998–9002.

Johannes F, Porcher E, Teixeira FK, Saliba-Colombani V, Simon M, Agier N, Bulski A, Albuisson J, Heredia F, Audigier P, *et al.* 2009. Assessing the impact of transgenerational epigenetic variation on complex traits. *PLoS Genetics* 5: e1000530.

Jung V, Violle C, Mondy C, Hoffmann L, Muller S. 2010. Intraspecific variability and trait-based community assembly. *The Journal of Ecology* 98: 1134–1140.

Kichenin E, Wardle DA, Peltzer DA, Morse CW, Freschet GT. 2013. Contrasting effects of plant inter- and intraspecific variation on community-level trait measures along an environmental gradient. *Functional Ecology* 27: 1254–1261.

van Kleunen M, Dawson W, Essl F, Pergl J, Winter M, Weber E, Kreft H, Weigelt P, Kartesz J, Nishino M, *et al.* 2015. Global exchange and accumulation of non-native plants. *Nature* 525: 100–103.

Kooke R, Johannes F, Wardenaar R, Becker F, Etcheverry M, Colot V, Vreugdenhil D, Keurentjes JJB. 2015. Epigenetic basis of morphological variation and phenotypic plasticity in *Arabidopsis thaliana*. *The Plant Cell* 27: 337–348.

Kuiper PJC, Bos M. 1992. *Plantago: A Multidisciplinary Study*. Springer.

Latzel V, Allan E, Bortolini Silveira A, Colot V, Fischer M, Bossdorf O. 2013. Epigenetic diversity increases the productivity and stability of plant populations. *Nature Communications* 4: 2875.

Lemaire G, Hodgson J, Moraes A, Carvalho PCF, Nabinger C. 2000. *Grassland Ecophysiology and Grazing Ecology*. CABI.

Lewis SL, Maslin MA. 2015. Defining the Anthropocene. *Nature* 519: 171–180.

Li Q, Eichten SR, Hermanson PJ, Springer NM. 2014. Inheritance patterns and stability of DNA methylation variation in maize near-isogenic lines. *Genetics* 196: 667–676.

Li X, Zhu J, Hu F, Ge S, Ye M, Xiang H, Zhang G, Zheng X, Zhang H, Zhang S, *et al.* 2012. Single-base resolution maps of cultivated and wild rice methylomes and regulatory roles of DNA methylation in plant gene expression. *BMC Genomics* 13: 300.

Linhart YB, Grant MC. 1996. Evolutionary significance of local genetic differentiation in plants. *Annual review of ecology and systematics* 27: 237–277.

Madritch M, Donaldson JR, Lindroth RL. 2006. Genetic identity of *Populus tremuloides* litter influences decomposition and nutrient release in a mixed forest stand. *Ecosystems* 9: 528–537.

McGill BJ, Enquist BJ, Weiher E, Westoby M. 2006. Rebuilding community ecology from functional traits. *Trends in Ecology & Evolution* 21: 178–185.

Mimura M, Yahara T, Faith DP, Vázquez-Domínguez E, Colautti RI, Araki H, Javadi F, Núñez-Farfán J, Mori AS, Zhou S, *et al.* 2017. Understanding and monitoring the consequences of human impacts on intraspecific variation. *Evolutionary Applications* 10: 121–139.

Mittell EA, Nakagawa S, Hadfield JD. 2015. Are molecular markers useful predictors of adaptive potential? *Ecology Letters* 18: 772–778.

Mundt CC. 2002. Use of multiline cultivars and cultivar mixtures for disease management. *Annual Review of Phytopathology* 40: 381–410.

Newbold T, Hudson LN, Hill SLL, Contu S, Lysenko I, Senior RA, Börger L, Bennett DJ, Choimes A, Collen B, *et al.* 2015. Global effects of land use on local terrestrial biodiversity. *Nature* 520: 45–50.

Niinemets Ü. 2010. A review of light interception in plant stands from leaf to canopy in different plant functional types and in species with varying shade tolerance. *Ecological Research* 25: 693–714.

Núñez-Farfán J, Schlichting CD. 2001. Evolution in changing environments: the 'synthetic' work of Clausen, Keck, and Hiesey. *The Quarterly Review of Biology* 76: 433–457.

Paaby AB, Rockman MV. 2014. Cryptic genetic variation: evolution's hidden substrate. *Nature reviews. Genetics* 15: 247–258.

Palumbi SR. 2001. Humans as the world's greatest evolutionary force. *Science* 293: 1786–1790.

Parmesan C. 2006. Ecological and evolutionary responses to recent climate change. *Annual Review of Ecology, Evolution, and Systematics* 37: 637–669.

Pons TL. 1992. Seed germination of *Plantago major* ssp. *major* and *Plantago lanceolata*. In: *Plantago: A Multidisciplinary Study*. Springer, 161–169.

Prieto I, Violle C, Barre P, Durand J-L, Ghesquiere M, Litrico I. 2015. Complementary effects of species and genetic diversity on productivity and stability of sown grasslands. *Nature Plants* 1: 15033.

Reed DH, Frankham R. 2001. How closely correlated are molecular and quantitative measures of genetic variation? A meta-analysis. *Evolution* 55: 1095–1103.

Reinders J, Wulff BBH, Mirouze M, Marí-Ordóñez A, Dapp M, Rozhon W, Bucher E, Theiler G, Paszkowski J. 2009. Compromised stability of DNA methylation and transposon immobilization in mosaic *Arabidopsis* epigenomes. *Genes & Development* 23: 939–950.

Reusch TBH, Ehlers A, Hämmerli A, Worm B. 2005. Ecosystem recovery after climatic extremes enhanced by genotypic diversity. *Proceedings of the National Academy of Sciences of the United States of America* 102: 2826–2831.

Richards CL, Alonso C, Becker C, Bossdorf O, Bucher E, Colomé-Tatché M, Durka W, Engelhardt J, Gaspar B, Gogol-Döring A, *et al.* 2017. Ecological plant epigenetics: Evidence from model and non-model species, and the way forward. *Ecology Letters* 20: 1576–1590.

Rotundo JL, Aguiar MR. 2008. Herbivory resistance traits in populations of *Poa ligularis* subjected to historically different sheep grazing pressure in Patagonia. *Plant Ecology* 194: 121–133.

Sagar GR, Harper JL. 1964. *Plantago major* L. *P. media* L. and *P. lanceolata* L. *The Journal of Ecology* 52: 189–221.

Sala OE, Chapin FS 3rd, Armesto JJ, Berlow E, Bloomfield J, Dirzo R, Huber-Sanwald E, Huenneke LF, Jackson RB, Kinzig A, *et al.* 2000. Global biodiversity scenarios for the year 2100. *Science* 287: 1770–1774.

Seymour DK, Koenig D, Hagmann J, Becker C, Weigel D. 2014. Evolution of DNA methylation patterns in the Brassicaceae is driven by differences in genome organization. *PLoS Genetics* 10: e1004785.

Siefert A, Violle C, Chalmandrier L, Albert CH, Taudiere A, Fajardo A, Aarssen LW, Baraloto C, Carlucci MB, Cianciaruso MV, *et al.* 2015. A global meta-analysis of the relative extent of intraspecific trait variation in plant communities. *Ecology Letters* 18: 1406–1419.

da Silveira Pontes L, Maire V, Schellberg J, Louault F. 2015. Grass strategies and grassland community responses to environmental drivers: a review. *Agronomy for Sustainable Development* 35: 1297–1318.

Smil V. 2013. *Harvesting the Biosphere*. MIT Press.

Snaydon RW. 1970. Rapid population differentiation in a mosaic environment. I. The response of *Anthoxanthum odoratum* populations to soils. *Evolution* 24: 257–269.

Stapledon RG. 1928. Cocksfoot grass (*Dactylis glomerata* L.): Ecotypes in relation to the biotic factor. *The Journal of Ecology* 16: 71–104.

Steffen W, Crutzen J, McNeill JR. 2007. The Anthropocene: are humans now overwhelming the great forces of Nature? *Ambio* 36: 614–621.

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.

Tilman D. 1985. The resource-ratio hypothesis of plant succession. *The American Naturalist* 125: 827–852.

Turesson G. 1922. The genotypical response of the plant species to the habitat. *Hereditas* 3: 211–350.

van Tienderen PH, van der Toorn J. 1991. Genetic differentiation between populations of *Plantago lanceolata*. L. Local adaptation in three contrasting habitats. *The Journal of Ecology* 79: 27–42.

Vergeer P, Rengelink R, Copal A. 2003. The interacting effects of genetic variation, habitat quality and population size on performance of *Succisa pratensis*. *Journal of Ecology* 91: 18–26.

Violle C, Enquist BJ, McGill BJ, Jiang L, Albert CH, Hulshof C, Jung V, Messier J. 2012. The return of the variance: intraspecific variability in community ecology. *Trends in Ecology & Evolution* 27: 244–252.

Violle C, Navas M-L, Vile D, Kazakou E, Fortunel C, Hummel I, Garnier E. 2007. Let the concept of trait be functional! *Oikos* 116: 882–892.

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. *The Journal of Ecology* 105: 1290–1297.

Warwick SI, Briggs D. 1978. The genecology of lawn weeds. I. Population differentiation in *Poa annua* L. in a mosaic environment of bowling green lawns and flower beds. *The New Phytologist* 81: 725–737.

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.

Weigel D, Colot V. 2012. Epialleles in plant evolution. *Genome Biology* 13: 249.

Westoby M. 1998. A leaf-height-seed (LHS) plant ecology strategy scheme. *Plant and Soil* 199: 213–227.

Wolff K, Van Delden W. 1987. Genetic analysis of ecological relevant morphological variability in *Plantago lanceolata* L. 1. Population characteristics. *Heredity* 58: 183–192.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornelissen JHC, Diemer M, *et al.* 2004. The worldwide leaf economics spectrum. *Nature* 428: 821–827.

Zhang Y-Y, Fischer M, Colot V, Bossdorf O. 2013. Epigenetic variation creates potential for evolution of plant phenotypic plasticity. *The New Phytologist* 197: 314–322.

Zhang H, Lang Z, Zhu J-K. 2018a. Dynamics and function of DNA methylation in plants. *Nature Reviews. Molecular Cell Biology* 19: 489–506.

Zhang Y-Y, Latzel V, Fischer M, Bossdorf O. 2018b. Understanding the evolutionary potential of epigenetic variation: a comparison of heritable phenotypic variation in epiRILs, RILs, and natural ecotypes of *Arabidopsis thaliana*. *Heredity* 121: 257–265.

Zopfi H-J. 1993. Ecotypic variation in *Rhinanthus alectorolophus* (Scopoli) Pollich (Scrophulariaceae) in relation to grassland management: II. The genotypic basis of seasonal ecotypes. *Flora* 188: 153–173.

Chapter II

Structure, stability and ecological significance of natural epigenetic variation: a large-scale survey in *Plantago lanceolata*

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New Phytologist

Research

Structure, stability and ecological significance of natural epigenetic variation: a large-scale survey in *Plantago lanceolata*

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Summary

• Within-species diversity is an important driver of ecological and evolutionary processes. Recent research has found that plants can harbour significant epigenetic diversity, but its extent, stability and ecological significance in natural populations is largely unexplored.

• We analysed genetic, epigenetic and phenotypic variation in a large number of natural grassland populations of *Plantago lanceolata*, covering a broad geographical and environmental range. Within-population diversity and among-population differentiation were calculated from genetic and epigenetic marker data and from measurements of phenotypic traits, both for plants in the field and for the F_1 generation grown in a common environment.

• We found weak but significant epigenetic population structure. A large part of the epigenetic population differences observed in the field was maintained in a common environment. Epigenetic differences were consistently related to genetic and environmental variation, and to a lesser degree to phenotypic variation and land use, with more grazed populations harbouring greater epigenetic diversity.

• Our study demonstrates that epigenetic diversity exists in natural populations of a common grassland species, and that at least part of this epigenetic diversity is stable, nonrandom and related to environmental variation. Experimental and more detailed molecular studies are needed to elucidate the mechanistic basis of these observed patterns.

Introduction

Within-species variation is an important level of biological diversity, sometimes with even stronger ecological effects than species-level variation (Des Roches *et al.*, 2018). In studies of natural populations, within-species variation has two main components: (1) the diversity within populations, which serves as the raw material for evolution and adaptation (Barrett & Schluter, 2008), and has been shown to contribute to the resistance and resilience of populations (Hughes *et al.*, 2008); and (2) the genetic differentiation among populations, which reflects local adaptation and other evolutionary processes such as drift and gene flow.

In the past, the study of intraspecific variation was mainly concerned with phenotypic or genetic differences among individuals or populations. In recent years, it has become clear that intraspecific variation also exists at the epigenetic level of DNA methylation or other epigenetic modifications of the genome. Epigenetic variation can be related to variation in phenotype (Cubas *et al.*, 1999; Cortijo *et al.*, 2014; Kooke *et al.*, 2015), and is therefore potentially relevant for ecology and evolutionary biology, as well as plant and animal breeding and conservation. Although much epigenetic variation is under genetic control, there are cases where epigenetic variation is independent of genetic variation, as a result of spontaneous epimutation (Becker *et al.*, 2011; Van Der Graaf *et al.*, 2015) or environmental induction (Jiang *et al.*, 2014; Quadrana & Colot, 2016), and it is particularly these cases where the study of epigenetic variation has the potential for true discovery of novel intraspecific differences and evolutionary potential (Bossdorf *et al.*, 2008; Richards *et al.*, 2017).

So far, in-depth documentation of intraspecific variation in DNA methylation has been largely restricted to some model plant species (e.g. *Arabidopsis thaliana, Oryza sativa* and *Zea mays*) with extensive genomic and epigenomic resources (Schmitz *et al.*, 2011, 2013; Becker *et al.*, 2011; Li *et al.*, 2012, 2014; Van Der Graaf *et al.*, 2015; Kawakatsu *et al.*, 2016). These studies documented substantial variation in the extent and stability of DNA methylation, both within genomes in different sequence contexts and genomic regions, and among different lines/genotypes and geographical origins. In addition, there has also been a notable increase in research on natural epigenetic variation in nonmodel species (Richards *et al.*, 2017). These studies confirmed that variation in DNA methylation is ubiquitous in natural populations, and that it usually exceeds DNA sequence variation when comparing populations from ecologically contrasting origins (Herrera & Bazaga, 2010; Lira-Medeiros *et al.*,

2010; Richards *et al.*, 2012; Medrano *et al.*, 2014; Schulz *et al.*, 2014). However, most of these previous studies included only few populations, often from very restricted geographical ranges, which makes their conclusions difficult to generalize across larger ranges of populations and environments.

Besides quantifying and describing epigenetic variation in wild populations, another important goal is to clarify and disentangle its relationships with genetic and phenotypic variation. In *A. thaliana*, much of the epigenomic variation appears to mirror underlying genetic patterns (Dubin *et al.*, 2015). However, the structure and dynamics of the *A. thaliana* epigenome are very unusual within the plant kingdom (Mirouze & Vitte, 2014; Alonso *et al.*, 2015) – with exceptionally low overall DNA methylation – and studies from nonmodel plants have reported patterns of epigenetic variation independent of genetic relatedness (Schulz *et al.*, 2014; Foust *et al.*, 2016; Gugger *et al.*, 2016). In addition, phenotypic variation can be caused by epigenetic differences *alone*, as it has been demonstrated, for instance, for flower symmetry, root length and flowering time (Cubas *et al.*, 1999; Cortijo *et al.*, 2014). In natural populations, significant correlations were found between epigenetic markers and several phenotypic traits (Herrera & Bazaga, 2010, 2013; Medrano *et al.*, 2014). However, these phenotypic measurements originated from the field, and so cannot disentangle the plastic and stable components of the relationship between epigenetic and phenotypic variation. In order to do so, epigenetic and phenotypic data must be compared between wild plants and their offspring in a common environment.

Apart from underlying genetic variation, heritable epigenetic variation also can be induced by environmental variability, as documented both in model and nonmodel species (Verhoeven *et al.*, 2010; Wibowo *et al.*, 2016). Because of the multiple sources of epigenetic variation (genetic, environmental and stochastic), its partial inheritance, and the multiple origins of phenotypic variation (genetic and epigenetic), teasing apart the relationships between these processes continues to be a challenge. However, some effects can be separated through experimental designs that combine field-collected and common-environment-derived material with environmental data (Bossdorf *et al.*, 2008). If epigenetic variation is correlated with environmental factors in the field, but this relationship disappears in the common environment, this indicates plastic responses, and their possible drivers might be found by relating the phenotypes from the field to the environmental variables. If the relationships are maintained in a common environment, then this indicates either natural selection acting on stable epigenetic variation or inheritance of environmentally induced epigenetic changes. Because environmentally induced epigenetic changes may be reset after few generations (Wibowo *et al.*, 2016), extending common garden studies across multiple generations can help to distinguish between these two cases. In any case, the environmental and phenotypic correlates of stable epigenetic variation can indicate the underlying drivers and targets of selection.

In summary, to understand the ecological and evolutionary significance of epigenetic variation, it is important to quantify epigenetic variation in large numbers of natural populations also in nonmodel plants and across broad geographical and environmental ranges, to couple epigenetic variation to genetic and phenotypic variation, and to combine field surveys with common garden approaches. Although the need for such studies was already identified a decade ago (Bossdorf *et al.*, 2008), we are not aware of any previous study that has addressed all of the questions above in a comprehensive way.

Here, we present a survey of natural epigenetic variation across 60 wild populations of *Plantago lanceolata*, a common and ecologically important plant species in Central European grasslands (Sagar & Harper, 1964). The studied populations covered a broad geographical and environmental range. We combined epigenetic with genetic and phenotypic data, and compared plants in the field with their F_1 offspring raised in a common environment to ask three research questions. (1) What is the extent, structure and stability of natural epigenetic variation in *P. lanceolata*? We expected significant epigenetic population structure, with differentiation among geographic regions and populations, and at least a partial persistence of the observed epigenetic variation in the common environment. (2) How is natural epigenetic variation related to genetic and phenotypic variation? We expected significant relationships between all three types of variation, with stronger genetic–epigenetic relationships in the glasshouse than in the field (as stable epigenetic variation is more likely genetically controlled), and stronger epigenetic–phenotypic relationships in the field, reflecting the plastic components of epigenetic and phenotypic variation. (3) How is natural epigenetic variation related to different environmental factors, in particular geographical and environmental distance and the intensities of land use in the studied grasslands? We expected epigenetic variation to show isolation by geographical and environmental distance, and a significant relationship with land-use intensity, all stronger in the field than in the glasshouse.

Materials and Methods

Study system

We worked with *Plantago lanceolata* L. (Plantaginaceae), a short-lived perennial rosette herb that is very common in European grasslands and grows under a wide range of environmental conditions. The species is a wind-pollinated and self-incompatible diploid (Kuiper & Bos, 1992) with a moderately sized genome ($1n \approx 1.28$ Gb). We studied natural populations of *P. lanceolata* within the German research platform Biodiversity Exploratories (www.biodiversity-exploratories.de), a large-scale and long-term project investigating relationships between land use, biodiversity and ecosystem processes (Fischer *et al.*, 2010). Among others, it comprises a hierarchical set of standardized grassland plots, with 50 plots in each of three regions (Fig. 1): the Schorfheide-Chorin Biosphere Reserve in the north, the Hainich National Park and surrounding areas in the middle, and the Schwäbische Alb Biosphere Reserve in the south of Germany, spanning across 600 km in total. In each region, the plots cover a wide range of land-use types and intensities, with precise data for the mowing, fertilization and grazing intensities of each plot, obtained from regular

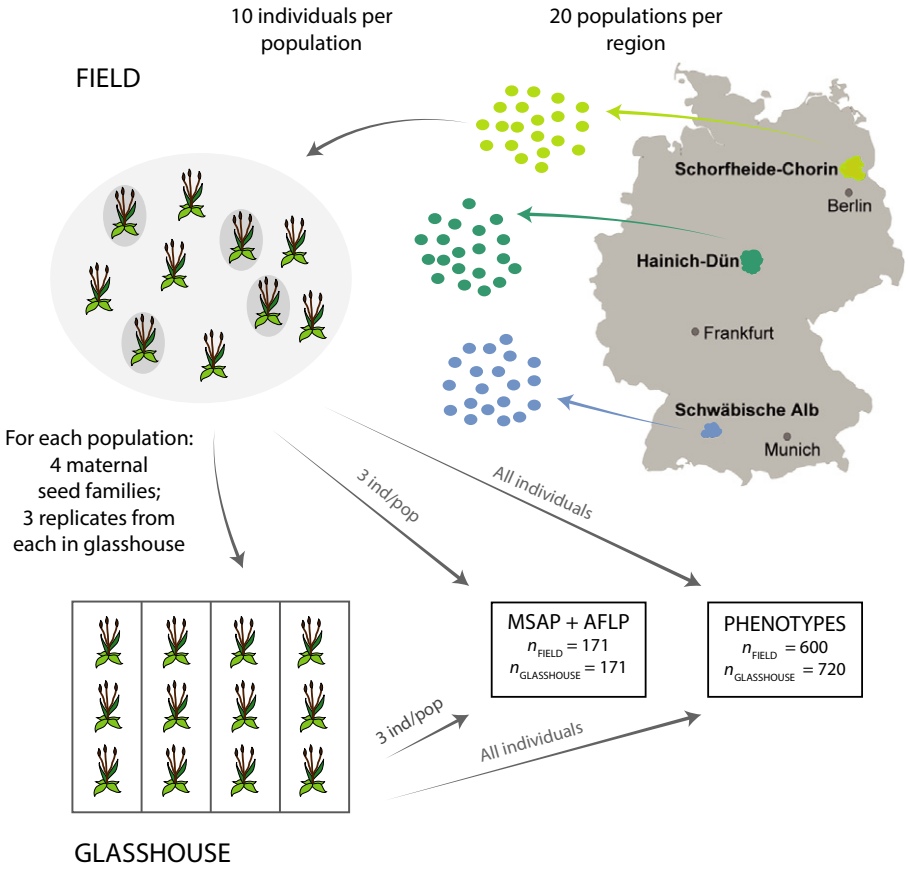

Fig. 1 Schematic of the experimental design of this study. We studied genetic, epigenetic and phenotypic variation in multiple populations of *Plantago lanceolata* in three regions of Germany, on plants growing in the field, as well as on their offspring grown in a common environment. A subset of the field individuals were used as parents for the F_1 generation, and three individuals per population from each environment were analysed for methylation sensitive amplified polymorphism (MSAP) and amplified fragment length polymorphism (AFLP) variation.

land-use inventories (Blüthgen *et al.*, 2012). *Plantago lanceolata* is one of the most common plant species in the Biodiversity Exploratories. Because of their large geographical extent and wealth of environmental data, these plots offer an excellent opportunity for studying epigenetic variation, and its ecological and environmental correlates, in natural plant populations.

Field survey

In September 2015, we collected leaf and seed material, and took phenotypic measurements in at least 20 grassland plots in each of the three regions. According to a vegetation survey from 2014, *P. lanceolata* occurred on 40, 38 and 27 plots in the three regions from south to north. To minimize the probability of management-related direct environmental induction of DNA methylation changes, we sampled only plots where at least 3 wk had passed after the last land-use event (mowing, fertilization or grazing), eventually limiting ourselves to 20 plots per region which maximized the land-use gradients as well as possible. Within regions, the sampled plots were on average 15 km apart from each other (Schwäbische Alb: mean = 11.2 km, range = 0.4–28.9 km; Hainich-Dün: mean = 14.5 km, range = 0.4–36.3 km; Schorfheide-Chorin: mean = 18.4 km, range = 0.4–42.5 km). We generally considered each plot a separate population.

In each population, we randomly selected at least 10 plant individuals along two parallel transects, altogether 615 individuals across the 60 populations. On each individual, we recorded plant height, length of the longest leaf, and the number of inflorescences, and we collected seeds for later common-garden cultivation (see in the next section 'Common garden study'). We then collected 2–3 undamaged leaves for molecular analyses and stored these at *c.* 5°C in a cooling box until being transferred to −20°C at the end of the day, and later freeze-dried them. We pressed three to eight leaves between blotting paper for later assessment of leaf traits. Upon returning from the field campaign, these leaves were dried in a drying oven at 70°C for at least 72 h, and they were weighed, and scanned with a flatbed photo scanner (Epson V600). The resulting images were analysed with ImageJ (Schneider *et al.*, 2012) to calculate average leaf aspect ratio and specific leaf area for each individual. We used the data from the five measured traits to calculate the phenotypic diversity for each population as the mean coefficient of variation (CV) of the five traits.

Common garden study

In order to obtain an F_1 generation of all studied populations, we sowed seeds of four randomly chosen maternal plants per population into seedling trays and stratified them at 5°C and under moist and dark conditions for 3 wk (Pons, 1992). After that, the trays were moved to a glasshouse with a 16 h : 8 h, day : night cycle at 21 : 15°C. The seeds then rapidly germinated, and we transplanted three seedlings per maternal family into 1-l pots filled with a standard potting soil. Altogether, we transplanted

741 seedlings. After 4 wk of growth, the pots were rearranged into a randomized block design, and were allowed to grow for another 6 wk. After that, we took two undamaged leaves per plant for the molecular analyses, flash-froze them in liquid nitrogen and subsequently freeze-dried them. We took the same phenotypic measurements as in the field populations, with five scanned leaves per plant for leaf trait measurements, and calculated the same population-level phenotypic diversities.

Molecular analyses

In order to assess genetic and epigenetic diversity and differentiation, we performed AFLP (amplified fragment length polymorphism) and MSAP (methylation-sensitive amplification polymorphism) analyses, respectively, on a total of 342 individuals (three regions × 19 populations × three individuals × two growing environments). For each population, we randomly chose three of the four maternal families in the glasshouse for the molecular analyses, and the same maternal families were used from the field samples (Fig. 1). In order to increase the accuracy of our analyses and exclude the possibility of a plate effect, we fitted all samples and technical replicates on one 384 multiwell PCR plate, thereby sacrificing one population from each region. Total genomic DNA was extracted from freeze-dried leaf tissue with the peqGOLD Plant DNA Mini Kit (VWR, Darmstadt, Germany). AFLP and MSAP laboratory and scoring procedures followed the protocols described in Schulz *et al.* (2014; Supporting Information Methods S1). We used four and eight selective primer combinations for AFLP and MSAP, respectively (Table S1). Comparison of the *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I reactions of MSAP analyses resulted in four different conditions for each fragment: (I) nonmethylated (band present in both parallel reactions), (II) CG-methylated (band only present in the *Eco*RI/*Msp*I reaction), and (III) CHG-hemimethylated restriction site (band only present in the *Eco*RI/*Hpa*II reaction), and (IV) an uninformative state with fragments absent in both reactions. We re-coded this data matrix – that contains three informative (I–III) and one uninformative condition (IV) at each locus – into three separate presence/absence matrices that correspond to the three informative conditions using the 'Mixed-Scoring 2' approach (Schulz *et al.*, 2013), thus making maximum use of the MSAP information. Furthermore, the two methylated conditions are methylated by two different enzymes – MET1 and CMT3, respectively – that are part of distinct molecular pathways (Law & Jacobsen, 2010) – and have different stabilities (II > III; see (Schmitz *et al.*, 2013), further supporting their separation. Overall error rates for AFLP and MSAP were 4.25% and 3.37%, respectively, based on 40 (12%) replicate samples each.

Data analysis

All analyses were done in R (R Development Core Team, 2008). To quantify genetic and epigenetic diversity, we calculated for each population Shannon's information index, and the number of polymorphic and private loci using the R script MSAP_CALC (Schulz *et al.*, 2013), based on AFLP or MSAP data. We analysed and visualized population structure through AMOVA and principal coordinates analysis (PCoA), using the POPPR and ADEGENET packages (Jombart & Ahmed, 2011; Kamvar *et al.*, 2015). As a measure of population differentiation, we used the population-level average Nei and Li distances (synonymous to the Soerensen–Dice and Bray–Curtis distances) from other populations calculated with the POPPR package. All analyses of MSAP data were run separately for each of the three MSAP subepiloci (MSAP-n, MSAP-m, MSAP-h), and in parallel for field and common-garden data (referred to as 'growing environment').

In order to assess the stability of epigenetic diversity and differentiation at the population level, we calculated regressions between the parental (field) and offspring (glasshouse) populations in these variables. In addition, we calculated locus-by-locus transmissibility of DNA methylation as described in Herrera *et al.* (2014), except that we excluded shared absences (cases of 'stability' where 0→0) because we considered them uninformative or misleading (there could be changes in other subepiloci at the same locus), and because the same information could otherwise be used multiple times in different datasets. For the AFLP data, we did not exclude the shared absences, considering that in that case there is only one binary data matrix.

In order to test for relationships between epigenetic, genetic and phenotypic variation, we calculated correlations between the respective Shannon diversities, and the population-level CV in the case of the phenotypes. To further explore relationships between these three levels of variation, while at the same time accounting for spatial autocorrelation (Legendre *et al.*, 2015), we employed redundancy analysis (RDA) combined with distance-based Moran's eigenvector maps (dbMEM; originally termed 'principal coordinates of neighbour matrices', PCNM). We used the VEGAN package in R (Oksanen *et al.*, 2017) to perform RDA and to obtain the spatial eigenfunctions. The dbMEM analysis resulted in six spatial eigenfunctions showing a positive spatial autocorrelation (positive eigenvalues), and we retained the three significant ones for further analyses. We then analysed the relationship between genetic and epigenetic variation both with and without correcting for spatial structure. Likewise, we tested the relationship between phenotypic and epigenetic variation in three different ways: first including only epigenetic data as explanatory variables, then including geographical structure, and finally including both geography and genetic variation. The latter allowed us to test whether there were any epigenotype–phenotype relationships independent from genetic variation. When genetic or epigenetic data were used as explanatory variable, we always used the first three PCoA axes of the respective datasets.

In order to test for relationships between epigenetic and genetic diversity and land-use intensity, we used general linear models that included genetic or epigenetic diversity as dependent variables, and tested for the effects of the different land-use components (mowing, fertilization and grazing), the effects of the regions, the growing environments (field vs glasshouse), and their interactions. Finally, we used RDA to test whether epigenetic variation was related to land-use intensity or other environmental variables, respectively, both with or without correcting for geographical structure via the dbMEM approach. For the

environmental variables, we included the following standardized environmental descriptors of the study plots: elevation, slope, aspect, mean height of vegetation, biomass per area, plant species richness and Shannon-diversity, as well as the Ellenberg indicator values for moisture, soil acidity and nutrients (F, R, N). For land-use intensity, we included the three land-use intensity components: mowing, fertilization and grazing, all taken from the Biodiversity Exploratories database (www.bexis.uni-jena.de). In order to account for multiple testing in the case of the RDAs, we report the false discovery rate-corrected *P*-values.

Results

Extent, structure and stability of epigenetic variation

The MSAP analysis of 326 individuals yielded 606 polymorphic epiloci, which were resolved into 1481 polymorphic subepiloci (560 n-type, 430 m-type and 491 h-type). AFLP analysis resulted in 545 polymorphic loci. The population-level epigenetic diversity was lower than genetic diversity, and decreased from n- to m- to h-subepiloci (Fig. 2a; Table S2). Population differentiation showed an opposite pattern, with the lowest interpopulation distances for AFLP, larger distances for MSAP n- and m-subepiloci, and strongest differentiation for MSAP h-subepiloci (Fig. 2b). These patterns also were visible in the PCoA, with increasing scatter of individuals from AFLP to MSAP n-, m- and h-subepiloci (Fig. 3). Although there was much overlap between the regions, there was a small degree of segregation, often with the Schwäbische Alb region most distinct from the other two. AMOVA confirmed that there was significant genetic and epigenetic differentiation between regions and populations in most cases, explaining around 2% of genetic variation (Fig. 3, Table S3). However, we found no significant differentiation for AFLP and MSAP-m in the glasshouse with AMOVA. The RDA/dbMEM analysis showed that there was significant spatial structure in all datasets; the amount of variance explained (ranging from 2.14% to 3.56%) generally decreased from the more stable epiloci towards the more unstable ones, and it was generally lower in the glasshouse than in the field (Table 1). In line with the AMOVA and PCoA results, one of the significant spatial eigenvectors separated the Schwäbische Alb region from the other two regions.

In order to assess the stability of the epigenetic differences observed in the field, we related the field-derived dataset to the glasshouse-derived data. The PCoA showed that the overall spread of individuals decreased in the glasshouse, but remained larger in MSAP-n, MSAP-m and particularly MSAP-h than in AFLP data. Moreover, differences among regions disappeared in AFLP and MSAP-m (Fig. 3). Comparison of descriptive parameters showed that the glasshouse-derived diversities followed the pattern of field data ($H'_{\mathrm{AFLP}} > H'_{\mathrm{MSAP\text{-}n}} > H'_{\mathrm{MSAP\text{-}m}} > H'_{\mathrm{MSAP\text{-}h}}$; Table S2). AFLP diversity was higher and MSAP-h diversity was lower in the glasshouse, and interpopulation distances were significantly lower in glasshouse in all cases (Fig. 2). In AMOVA, the regional components of variance were generally maintained in the glasshouse, albeit at a slightly lower level, whereas population components were not significant anymore for AFLP and MSAP-m (Fig. 3). The comparison of parent and offspring populations for genetic and epigenetic diversity and differentiation data showed significant stability in all MSAP conditions but not in AFLP (Fig. 4; Table S4a). Locus-by-locus transmissibility was highest in the AFLP markers (86%) and decreased from MSAP-n (57%) to MSAP-m (52%) and MSAP-h (40%) (Table S4b).

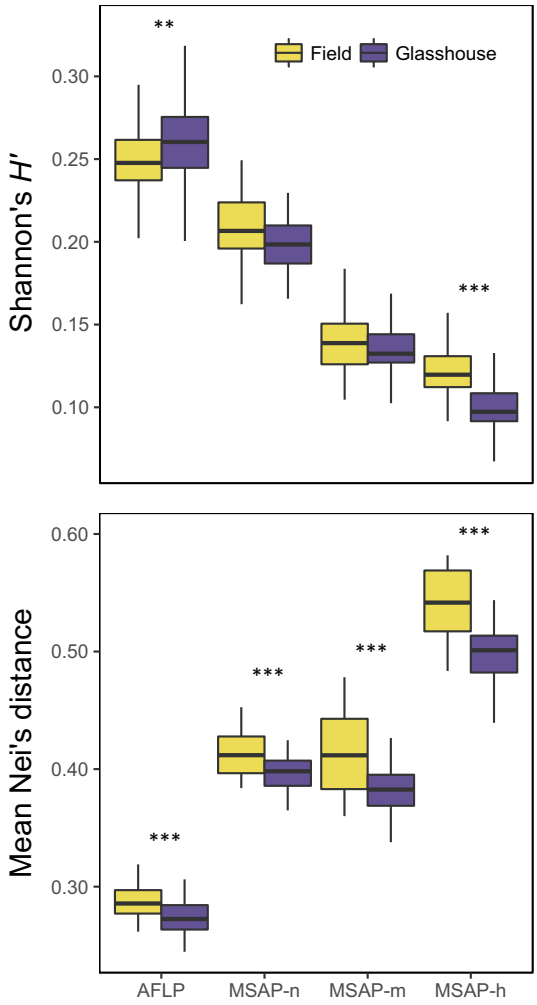

Fig. 2 Magnitudes of epigenetic and genetic variation among 60 natural *Plantago lanceolata* populations. Shannon diversity (*H'*, upper panel) and mean Nei's distances between populations (lower panel) were compared between amplified fragment length polymorphism (AFLP) markers and the three methylation sensitive amplified polymorphism (MSAP) conditions (nonmethylated, methylated and hemimethylated subepiloci), for plants in the field and their offspring grown in a common glasshouse environment. The boxplots indicate medians, 25th/75th percentiles, and the 1.5 × interquartile range. Significant differences between field and glasshouse plants, based on permutation tests with 10^4 replications: **, $P < 0.01$; ***, $P < 0.001$.

Relationships between epigenetic, genetic and phenotypic variation

At the level of aggregated, population-level measures of diversity, there were no significant relationships between epigenetic diversity and genetic or phenotypic diversity, respectively (Table S5).

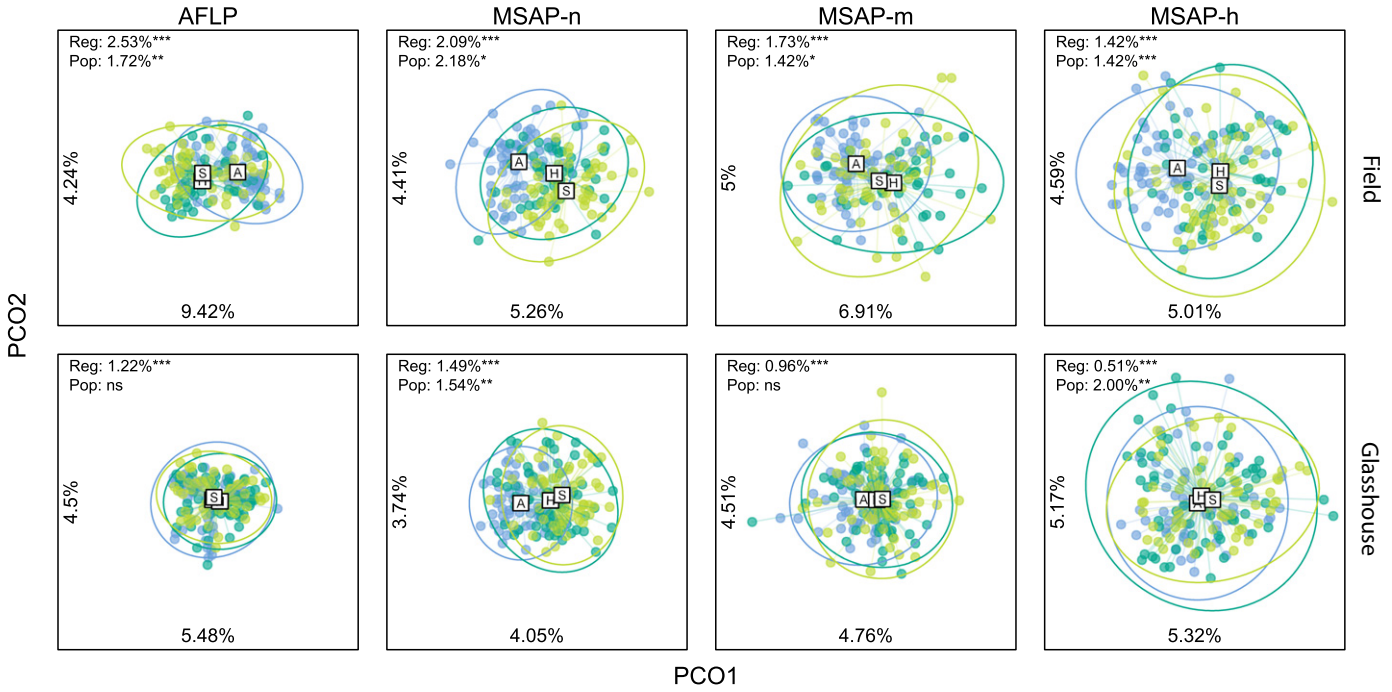

Fig. 3 Epigenetic and genetic variation among *Plantago lanceolata* individuals in the field (upper row) and glasshouse (lower row). We show principal coordinates analyses (PCoA) for genetic (AFLP, amplified fragment length polymorphism) and epigenetic (MSAP, methylation sensitive amplified polymorphism) markers, separately for nonmethylated (MSAP-n), methylated (MSAP-m) and hemimethylated (MSAP-h) subepiloci. The three regions are distinguished by colour (A-Alb, blue; H-Hainich, green; S-Schorfheide, yellow), with their centroid marked by the respective abbreviation, and the coloured ellipses delineating the 95% bivariate confidence interval around their mean. The percentages on the axes indicate the amount of variance explained by each PCoA axis. In addition, the amounts of variance assigned by AMOVA to region and populations are given in the upper left corner of each panel, with significances marked as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Table 1 Results of redundancy analyses (RDA) relating epigenetic variation in *Plantago lanceolata* to genetic (GEN) or phenotypic (PHEN) variation, as well as to spatial geographic (GEO) variation, environmental (ENV) or land-use intensity (LUI) variation, separately for the three different methylation sensitive amplified polymorphism (MSAP) epiloci types, and for field vs glasshouse data.

	Field						Glasshouse					
	MSAP-n		MSAP-m		MSAP-h		MSAP-n		MSAP-m		MSAP-h	
	Var %	*P*	Var %	*P*	Var %	*P*	Var %	*P*	Var %	*P*	Var %	*P*
EPI vs GEO	3.56	0.003	3.26	0.003	3.09	0.003	3.00	0.003	2.48	0.008	2.14	0.010
EPI vs GEN	3.48	0.003	3.62	0.003	3.28	0.003	2.11	0.003	2.17	0.008	1.95	0.158
EPI vs GEN (GEO)	2.80	0.003	3.12	0.003	2.75	0.003	2.00	0.034	2.15	0.008	1.99	0.158
PHEN vs EPI	4.81	0.320	3.20	0.736	7.43	0.031	3.48	0.494	2.45	0.831	4.47	0.235
PHEN vs EPI (GEO)	4.26	0.420	3.99	0.587	8.52	0.023	3.68	0.494	2.51	0.831	4.44	0.235
PHEN vs EPI (GEO + GEN)	5.15	0.320	4.27	0.587	8.41	0.023	4.36	0.349	3.42	0.791	4.50	0.235
EPI vs ENV	8.28	0.003	7.94	0.003	7.71	0.003	6.79	0.003	6.50	0.008	6.35	0.040
EPI vs ENV (GEO)	7.13	0.072	7.27	0.030	6.96	0.170	6.03	0.386	5.94	0.791	6.20	0.235
EPI vs LUI	2.26	0.012	2.28	0.030	2.26	0.023	1.97	0.023	1.77	0.791	1.84	0.235
EPI vs LUI (GEO)	2.11	0.320	2.12	0.326	2.25	0.043	1.88	0.245	1.74	0.791	1.89	0.235

(GEO) or (GEO + GEN) indicate whether the effects of spatial or genetic structure were accounted for before testing a specific relationship. The values are the % variances explained by each model, followed by their FDR-corrected significance levels. *P*-values < 0.05 highlighted in grey. MSAP-n, nonmethylated; MSAP-m, methylated; MSAP-h, hemimethylated.

However, when we analysed the individual-level relationships between the three types of variation through RDA, we found that except for the hemimethylated loci in the glasshouse, epigenetic and genetic variation were generally significantly related (Table 1). When spatial autocorrelation was included, the amounts of variance explained decreased, but the relationships remained significant. Moreover, the variance explained was generally lower in the glasshouse than in the field.

In contrast to epigenetic–genetic relationships, there was little evidence of relationships between epigenetic and phenotypic variation. Only for MSAP-h loci in the field, phenotypic variation was significantly related to epigenetic variation, and this

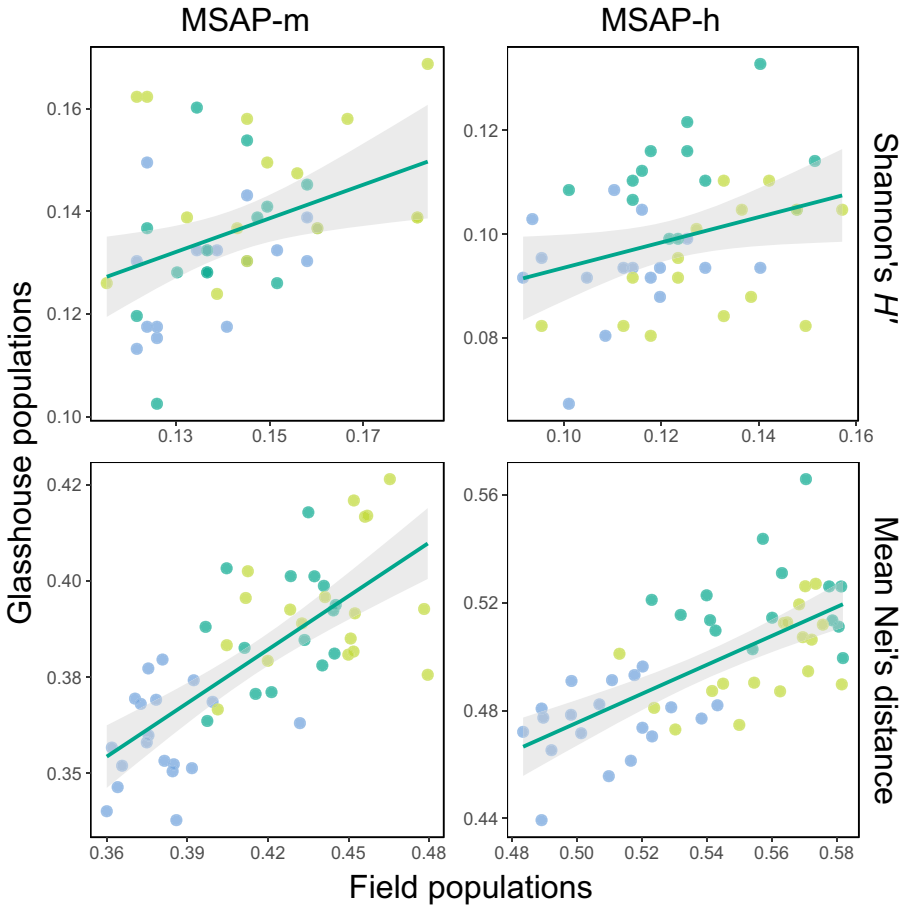

Fig. 4 Stability of epigenetic variation in natural populations of *Plantago lanceolata*. We show relationships between the epigenetic diversities and mean epigenetic distances of 60 wild populations and their glasshouse-grown offspring, separately for methylated (MSAP-m) and hemimethylated (MSAP-h) loci. The three regions are distinguished by colour (Alb, blue; Hainich, green; Schorfheide, yellow). The fitted generalized linear models (GLMs) and 95% confidence intervals are shown as solid lines and grey shading, respectively. All four regressions are significant at $P \leq 0.01$.

relationship remained significant also after incorporating spatial and genetic structure into the model.

Environmental correlates

We found a significant positive relationship between grazing intensity and MSAP-m diversity in both field and glasshouse plants (grazing main effect: $F = 11.6$, $P = 0.001$), and a significant grazing-by-growing-environment interaction for MSAP-h diversity ($F = 7.35$, $P = 0.008$), where a positive correlation was present in the field but disappeared in the glasshouse (Fig. 5; Table S6). In addition, there also was a significant main effect of mowing on MSAP-h diversity ($F = 5.20$, $P = 0.025$), and a mowing-by-region interaction for MSAP-n diversity ($F = 5.98$; $P = 0.004$) (Table S6). We found no significant land-use effects in the analysis of genetic diversity.

Epigenetic variation was not only related to land use, it also was significantly related to other environmental factors for all MSAP loci types in the field and glasshouse. In the RDA analyses, environmental descriptors explained some 6–8% of the epigenetic variation, following the same pattern as before: the variation explained decreased from the more stable towards the more unstable epiloci and was lower in the glasshouse than in the field (Table 1). However, when spatial structure was included in the model, only the relationship with MSAP-m in the field remained significant. Epigenetic variation was also related to land use in the RDAs, with significant relationships in the field for all MSAP epiloci and in the glasshouse for MSAP-n. When geographical structure was included in the models, the only remaining significant relationship was the one including the MSAP-h epiloci in the field.

Discussion

The ecological and evolutionary role of epigenetic variation in natural plant populations has received much attention in recent years. Here, we studied the extent, structure and stability of epigenetic diversity and differentiation, and its genetic, phenotypic and environmental correlates, in a large number of natural populations of *Plantago lanceolata*. We found low levels of epigenetic variation and population structure, and a partly stable transmission of the signal into the next generation. The heritable part of the epigenetic variation was consistently related to genetic and environmental variation, and to the land-use intensity in the studied grasslands, whereas the nonheritable part was associated also with plant phenotype.

Extent, structure and stability of epigenetic variation

We found that overall levels of within-population epigenetic diversity were rather moderate in natural populations of *P. lanceolata*, and that the values for epigenetic diversity were

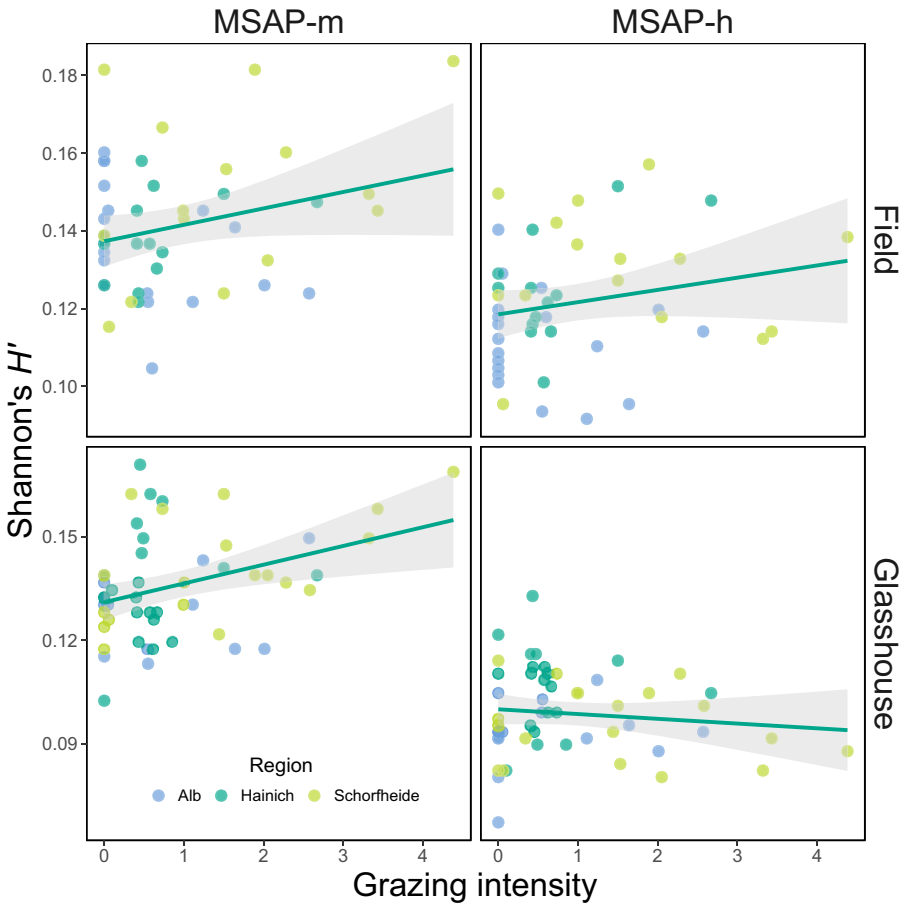

Fig. 5 Relationships between grazing intensity and epigenetic diversity in 60 grassland populations of *Plantago lanceolata*, separately for the methylated (MSAP-m) and hemimethylated (MSAP-h) methylation sensitive amplified polymorphism (MSAP) conditions. Epigenetic diversity increases with grazing intensity, and the pattern is stable in the methylated, but not in the hemimethylated condition. The fitted generalized linear models (GLMs) and 95% confidence intervals are shown as solid lines and grey shading, respectively.

generally lower than for genetic diversity. This is in contrast to several previous studies which found higher epigenetic than genetic diversity (Herrera & Bazaga, 2010; Lira-Medeiros *et al.*, 2010; Richards *et al.*, 2012, 2017; Medrano *et al.*, 2014; Schulz *et al.*, 2014). It is possible that this is mainly driven by the relatively high within-population genetic diversity. *Plantago lanceolata* is wind-pollinated and an obligate outcrosser, and these characteristics, together with enhanced dispersal through livestock and vehicles in the studied semi-agricultural landscapes, most probably result in high gene flow (which also is indicated by the low levels of population differentiation, the high number of polymorphic loci and extremely low number of private loci in populations). This in turn maintains higher diversity in the more stable amplified fragment length polymorphism (AFPL) loci, whereas the less stable methylation-sensitive amplification polymorphism (MSAP) loci are partly homogenized within populations by the common environmental conditions. This idea is supported by the differences between MSAP subepiloci types, where within-population diversity decreases from the more stable nonmethylated to the less stable methylated and hemimethylated conditions.

Surprisingly, we found that genetic diversity (within populations) was significantly higher and genetic differentiation (among populations) was lower in the glasshouse than in the field. A possible explanation is that we established the glasshouse experiment from randomly selected seedlings, which, unlike their mother plants, had not undergone any selection, thus resulting in a higher diversity in the F_1 generation.

Epigenetic differentiation between regions and populations was generally low but nevertheless significant in all cases except for population differentiation of MSAP-m in the glasshouse. In contrast to the results with within-population diversity, population differentiation was generally larger at the epigenetic level than at the genetic level, with highest values for the least stable (MSAP-h) markers. Again, these results are consistent with the idea that epigenetic variation is generally more responsive to environmental conditions, which on the one hand decreases diversity within populations but at the same time increases divergence between natural populations, relative to genetic variation.

The epigenetic differentiation observed in the field was not just a result of short-term environmental induction, but much of it was stably transmitted to the F_1 offspring, as shown by the analysis of glasshouse data, the parent–offspring population comparisons, the locus-by-locus transmissibility analyses, and the redundancy analyses including spatial eigenvectors. Although population differentiation generally decreased in glasshouse plants, it remained substantially larger at the epigenetic than the genetic level, with strongest differentiation in MSAP-h markers. In general, hemimethylated MSAP-h loci (reflecting CHG sequence context) appear to be more responsive to the environment than methylated MSAP-m loci, but they also lose their differences again more rapidly in a common environment. The

transmissibility of DNA methylation observed in the different conditions was in concordance with previous results from *A. thaliana* (Schmitz *et al.*, 2013; Van Der Graaf *et al.*, 2015).

To our knowledge, our study constitutes the first rigorous test of the stability of natural epigenetic variation through comparison of wild plants and their common-garden offspring in a sexually reproducing nonmodel plant. Gao *et al.* (2010) compared field and common-garden populations of the invasive alligator weed (*Alternanthera philoxeroides*), but with vegetatively propagated material originating from only three contrasting habitats. The authors found a very low level of variation (*c.* 5% polymorphic MSAP loci), but nevertheless 22% of the polymorphic loci were transmitted from field to common garden. Other studies quantified the heritability of stress-induced changes in a controlled environment (Verhoeven *et al.*, 2010), or sporophyte-to-pollen transmissibility of DNA methylation in the field (Herrera *et al.*, 2014), all without including both field and common garden populations. In summary, we found weak but significant natural epigenetic population structure, and part of the population differences in epigenetic diversity were maintained in a common environment.

Relationships between epigenetic, genetic and phenotypic variation

Besides characterizing the extent, structure and stability of natural epigenetic variation in itself, another major goal is to understand the (genetic and environmental) origins of this variation and its ecological and evolutionary consequences. Here, we found consistent significant relationships between epigenetic and genetic variation, as well as some – albeit weaker – evidence for a relationship between epigenetic and phenotypic variation.

Depending on MSAP epilocus type and growing environment, the genetic variation among *Plantago* individuals explained 2–3% of the epigenetic variation in our study. Other field studies in nonmodel plants (Herrera & Bazaga, 2010; Schulz *et al.*, 2014; Foust *et al.*, 2016) found no relationships between epigenetic and genetic variation at all, whereas (usually controlled-environment) studies in the model plant *A. thaliana* generally showed strong genetic control of DNA methylation variation (Dubin *et al.*, 2015; Kawakatsu *et al.*, 2016), which led to debate about the true epigenetic nature of DNA methylation. Although the previous nonmodel studies might have missed true genetic–epigenetic relationships because of the few MSAP and AFLP markers they used, or because of their less controlled environmental conditions which created additional stochastic and environmentally induced epigenetic 'noise' and thereby made it more difficult to detect such relationships, another explanation could be that epimutation rates in these species also could be several orders of magnitude greater than genetic mutation rates, as it has been shown in *A. thaliana* (Schmitz *et al.*, 2011), and thus the two types of variations diverged. However, the strong epigenetic–genetic association in *A. thaliana* could be due to its unusual genomic and epigenomic characteristics (i.e. small genome size and low global DNA methylation (Alonso *et al.*, 2015), transposable elements (TEs) and DNA methylation concentrated around the centromeres). Most other plants have larger genomes with more TEs and DNA methylation along the whole chromosomes (Mirouze & Vitte, 2014) making it very difficult to extrapolate from *A. thaliana* to other species. It seems plausible that in the majority of plants the truth lies somewhere in between, with a strong genetic control of epigenetic variation but also some level of independence of it. Here, we found some genetic–epigenetic associations, but the total amount of epigenetic variation explained by genetic variation remained low, most likely a consequence of the extremely high degree of heterozygosity of the *P. lanceolata* genome (A.-L. Laine, pers. comm.), mirrored by the high polymorphism of AFLP loci and low variation among populations, and of the low resolution of MSAP and AFLP markers, even though the number of markers used was close to the upper limit of feasibility for these methods.

We also found some association between epigenetic and phenotypic variation. In the field data, some 8% of the combined variation in five of the phenotypic traits could be explained by variation in MSAP-h, the most unstable type of MSAP epiloci, even after correcting for geographical and genetic variation. However, these patterns were absent in glasshouse data, which suggests that some of the phenotypic responses by which these plants respond to environmental variation in the field might be associated with underlying reversible DNA methylation changes. Other studies in wild nonmodel populations also found natural epigenetic and phenotypic variation to be related (Herrera & Bazaga, 2010, 2013; Medrano *et al.*, 2014), and studies with *A. thaliana* epiRILs demonstrated a mechanistic relationship between epigenetic variation and phenotypic variation (Cortijo *et al.*, 2014; Kooke *et al.*, 2015). In the former ones, no common-garden measurements were part of the design, whereas in the *Arabidopsis* studies the epigenetic–phenotypic relationships proved to be heritable over several generations. From our data this does not seem to be the case in *P. lanceolata*, which is infamous for its high phenotypic plasticity (Warwick & Briggs, 1979), as well as high gene flow and heterozygosity.

Environmental correlates

Whether epigenetic variation is plastic, environmentally induced, or stable, ultimately only a significant relationship with the environment is proof that the observed variation is ecologically significant (Bossdorf *et al.*, 2008). However, testing for such environmental correlates in a large population sample requires high-quality environmental data for all studied populations. We were fortunate to be able to use the rich metadata from the Biodiversity Exploratories, which allowed us not only to relate epigenetic to geographical and environmental variation, but also to test the effects of mowing, fertilization and grazing intensity – land-use processes that play a key role in the studied grasslands (Fischer *et al.*, 2010; Blüthgen *et al.*, 2012). We found a consistent and stable relationship between epigenetic and environmental variation that was maintained in the F_1 generation in a common environment, suggesting that at least part of the observed epigenetic variation might be related to environmental adaptation of *P. lanceolata*. The epigenetic-environmental

relationships mostly disappeared after spatial structure was incorporated into the models which indicates that not only epigenetic variation, but also environmental factors were spatially autocorrelated and likely co-varied in space.

Epigenetic variation also was related to land use, albeit to a much lesser degree than to other environmental variables. We found grazing intensity to be positively related to epigenetic diversity in the field. A possible explanation for this is that grazing creates environmental heterogeneity which results in variable epigenetic signatures of plant individuals. In contrast to mowing and fertilization, which are applied rather homogeneously within managed grasslands, grazing is a spatially heterogeneous process, with irregular trampling patterns, selective removal of biomass, and patchy deposition of nutrients from animal droppings (Bakker *et al.*, 1984; Adler *et al.*, 2001; Socher *et al.*, 2013). The relationship was plastic – not maintained in the glasshouse – for the hemimethylated MSAP loci, but it was stable for the methylated loci, consistent with the different stabilities of the two subepilocus types. Together, these results suggest that the grazing-related MSAP-h variation might reflect plastic phenotypic responses of *Plantago lanceolata* to land use, whereas the MSAP-m variation might reflect past selection on stable epigenetic variation, and thus adaptive epigenetic differentiation in these plant populations.

Of course, we only studied one offspring generation, so we cannot distinguish between environmentally induced, transient heritability – as has been found, for example, in *A. thaliana* (Wibowo *et al.*, 2016) – from truly stable epigenetic variation. Nevertheless, our study is the first demonstration of stable environment–epigenetics relationship in natural populations of a sexually reproducing nonmodel plant, and it is particularly intriguing that we observed this relationship only between grazing intensity and epigenetic but not genetic variation, demonstrating that at least sometimes epigenetic variation has the potential to provide truly novel insights.

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Author contributions

OB and WD planned and designed the research; BG conducted fieldwork, performed experiments and laboratory work; and all authors analysed the data and wrote the manuscript.

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References

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

Alonso C, Pérez R, Bazaga P, Herrera CM. 2015. Global DNA cytosine methylation as an evolving trait: phylogenetic signal and correlated evolution with genome size in angiosperms. *Frontiers in Genetics* **6**: 4.

Bakker JP, de Leeuw J, van Wieren SE. 1984. Micro-patterns in grassland vegetation created and sustained by sheep-grazing. *Vegetatio* **55**: 153–161.

Barrett RDH, Schluter D. 2008. Adaptation from standing genetic variation. *Trends in Ecology & Evolution* **23**: 38–44.

Becker C, Hagmann J, Müller J, Koenig D, Stegle O, Borgwardt K, Weigel D. 2011. Spontaneous epigenetic variation in the *Arabidopsis thaliana* methylome. *Nature* **480**: 245–249.

Blüthgen N, Dormann CF, Prati D, Klaus VH, Kleinebecker T, Hölzel N, Alt F, Boch S, Gockel S, Hemp A *et al.* 2012. A quantitative index of land-use intensity in grasslands: integrating mowing, grazing and fertilization. *Basic and Applied Ecology* **13**: 207–220.

Bossdorf O, Richards CL, Pigliucci M. 2008. Epigenetics for ecologists. *Ecology Letters* **11**: 106–115.

Cortijo S, Wardenaar R, Colomé-Tatché M, Gilly A, Etcheverry M, Labadie K, Caillieux E, Hospital F, Aury J-M, Wincker P *et al.* 2014. Mapping the epigenetic basis of complex traits. *Science* **343**: 1145–1148.

Cubas P, Vincent C, Coen E. 1999. An epigenetic mutation responsible for natural variation in floral symmetry. *Nature* **401**: 157–161.

Des Roches S, Post DM, Turley NE, Bailey JK, Hendry AP, Kinnison MT, Schweitzer JA, Palkovacs EP. 2018. The ecological importance of intraspecific variation. *Nature Ecology & Evolution* **2**: 57–64.

Dubin MJ, Zhang P, Meng D, Remigereau M-S, Osborne EJ, Casale FP, Drewe P, Kahles A, Jean G, Vilhjálmsson B *et al.* 2015. DNA methylation in *Arabidopsis* has a genetic basis and shows evidence of local adaptation. *eLife* **4**: e05255.

Fischer M, Bossdorf O, Gockel S, Hänsel F, Hemp A, Hessenmöller D, Korte G, Nieschulze J, Pfeiffer S, Prati D *et al.* 2010. Implementing large-scale and long-term functional biodiversity research: the Biodiversity Exploratories. *Basic and Applied Ecology* **11**: 473–485.

Foust CM, Preite V, Schrey AW, Alvarez M, Robertson MH, Verhoeven KJF, Richards CL. 2016. Genetic and epigenetic differences associated with environmental gradients in replicate populations of two salt marsh perennials. *Molecular Ecology* **25**: 1639–1652.

Gao L, Geng Y, Li B, Chen J, Yang J. 2010. Genome-wide DNA methylation alterations of *Alternanthera philoxeroides* in natural and manipulated habitats:

implications for epigenetic regulation of rapid responses to environmental fluctuation and phenotypic variation. *Plant, Cell & Environment* **33**: 1820–1827.

Gugger PF, Fitz-Gibbon S, Pellegrini M, Sork VL. 2016. Species-wide patterns of DNA methylation variation in *Quercus lobata* and their association with climate gradients. *Molecular Ecology* **25**: 1665–1680.

Herrera CM, Bazaga P. 2010. Epigenetic differentiation and relationship to adaptive genetic divergence in discrete populations of the violet *Viola cazorlensis*. *New Phytologist* **187**: 867–876.

Herrera CM, Bazaga P. 2013. Epigenetic correlates of plant phenotypic plasticity: DNA methylation differs between prickly and nonprickly leaves in heterophyllous *Ilex aquifolium* (Aquifoliaceae). *Botanical Journal of the Linnean Society* **171**: 441–452.

Herrera CM, Medrano M, Bazaga P. 2014. Variation in DNA methylation transmissibility, genetic heterogeneity and fecundity-related traits in natural populations of the perennial herb *Helleborus foetidus*. *Molecular Ecology* **23**: 1085–1095.

Hughes AR, Inouye BD, Johnson MTJ, Underwood N, Vellend M. 2008. Ecological consequences of genetic diversity. *Ecology Letters* **11**: 609–623.

Jiang C, Mithani A, Belfield EJ, Mott R, Hurst LD, Harberd NP. 2014. Environmentally responsive genome-wide accumulation of *de novo Arabidopsis thaliana* mutations and epimutations. *Genome Research* **24**: 1821–1829.

Jombart T, Ahmed I. 2011. adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. *Bioinformatics* **27**: 3070–3071.

Kamvar ZN, Brooks JC, Grünwald NJ. 2015. Novel R tools for analysis of genome-wide population genetic data with emphasis on clonality. *Frontiers in Genetics* **6**: 208.

Kawakatsu T, Huang S-SC, Jupe F, Sasaki E, Schmitz RJ, Urich MA, Castanon R, Nery JR, Barragan C, He Y *et al.* 2016. Epigenomic diversity in a global collection of *Arabidopsis thaliana* accessions. *Cell* **166**: 492–505.

Kooke R, Johannes F, Wardenaar R, Becker F, Etcheverry M, Colot V, Vreugdenhil D, Keurentjes JJB. 2015. Epigenetic basis of morphological variation and phenotypic plasticity in *Arabidopsis thaliana*. *Plant Cell* **27**: 337–348.

Kuiper PJC, Bos M. 1992. *Plantago: a multidisciplinary study*. Berlin, Germany: Springer Science & Business Media.

Law JA, Jacobsen SE. 2010. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nature Reviews. Genetics* **11**: 204–220.

Legendre P, Fortin M-J, Borcard D. 2015. Should the Mantel test be used in spatial analysis? (P Peres-Neto, Ed.). *Methods in Ecology and Evolution/British Ecological Society* **6**: 1239–1247.

Li Q, Eichten SR, Hermanson PJ, Springer NM. 2014. Inheritance patterns and stability of DNA methylation variation in maize near-isogenic lines. *Genetics* **196**: 667–676.

Li X, Zhu J, Hu F, Ge S, Ye M, Xiang H, Zhang G, Zheng X, Zhang H, Zhang S *et al.* 2012. Single-base resolution maps of cultivated and wild rice methylomes and regulatory roles of DNA methylation in plant gene expression. *BMC Genomics* **13**: 300.

Lira-Medeiros CF, Parisod C, Fernandes RA, Mata CS, Cardoso MA, Ferreira PCG. 2010. Epigenetic variation in mangrove plants occurring in contrasting natural environment. *PLoS ONE* **5**: e10326.

Medrano M, Herrera CM, Bazaga P. 2014. Epigenetic variation predicts regional and local intraspecific functional diversity in a perennial herb. *Molecular Ecology* **23**: 4926–4938.

Mirouze M, Vitte C. 2014. Transposable elements, a treasure trove to decipher epigenetic variation: insights from *Arabidopsis* and crop epigenomes. *Journal of Experimental Botany* **65**: 2801–2812.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P *et al.* 2017. vegan: Community Ecology Package. 2.5-2 [WWW document] URL https://cran.ism.ac.jp/web/packages/vegan/vegan.pdf [accessed 5 September 2017].

Pons TL. 1992. Seed germination of *Plantago major ssp. major* and *Plantago lanceolata*. In: Kuiper PJC, Bos M, eds. Plantago: *a multidisciplinary study*. Berlin, Germany: Springer, 161–169.

Quadrana L, Colot V. 2016. Plant transgenerational epigenetics. *Annual Review of Genetics* **50**: 467–491.

R Development Core Team. 2008. *R: a language and environment for statistical computing*. Vienna, Austria: R Development Core Team.

Richards CL, Alonso C, Becker C, Bossdorf O, Bucher E, Colomé-Tatché M, Durka W, Engelhardt J, Gaspar B, Gogol-Döring A *et al.* 2017. Ecological plant epigenetics: evidence from model and non-model species, and the way forward. *Ecology Letters* **20**: 1576–1590.

Richards CL, Schrey AW, Pigliucci M. 2012. Invasion of diverse habitats by few Japanese knotweed genotypes is correlated with epigenetic differentiation. *Ecology Letters* **15**: 1016–1025.

Sagar GR, Harper JL. 1964. *Plantago major* L., *P. media* L. and *P. lanceolata* L. *Journal of Ecology* **52**: 189–221.

Schmitz RJ, Schultz MD, Lewsey MG, O'Malley RC, Urich MA, Libiger O, Schork NJ, Ecker JR. 2011. Transgenerational epigenetic instability is a source of novel methylation variants. *Science* **334**: 369–373.

Schmitz RJ, Schultz MD, Urich MA, Nery JR, Pelizzola M, Libiger O, Alix A, McCosh RB, Chen H, Schork NJ *et al.* 2013. Patterns of population epigenomic diversity. *Nature* **495**: 193–198.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**: 671–675.

Schulz B, Eckstein RL, Durka W. 2013. Scoring and analysis of methylation-sensitive amplification polymorphisms for epigenetic population studies. *Molecular Ecology Resources* **13**: 642–653.

Schulz B, Eckstein RL, Durka W. 2014. Epigenetic variation reflects dynamic habitat conditions in a rare floodplain herb. *Molecular Ecology* **23**: 3523–3537.

Socher SA, Prati D, Boch S, Müller J, Baumbach H, Gockel S, Hemp A, Schöning I, Wells K, Buscot F *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.

Van Der Graaf A, Wardenaar R, Neumann DA, Taudt A, Shaw RG, Jansen RC, Schmitz RJ, Colomé-Tatché M, Johannes F. 2015. Rate, spectrum, and evolutionary dynamics of spontaneous epimutations. *Proceedings of the National Academy of Sciences, USA* **112**: 6676–6681.

Verhoeven KJF, Jansen JJ, van Dijk PJ, Biere A. 2010. Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytologist* **185**: 1108–1118.

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. *New Phytologist* **83**: 509–536.

Wibowo A, Becker C, Marconi G, Durr J, Price J, Hagmann J, Papareddy R, Putra H, Kageyama J, Becker J *et al.* 2016. Hyperosmotic stress memory in *Arabidopsis* is mediated by distinct epigenetically labile sites in the genome and is restricted in the male germline by DNA glycosylase activity. *eLife* **5**: e13546.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Methods S1 AFLP and MSAP protocol, adapted from Schulz *et al.* (2014).

Table S1 Adaptor- and primer sequences used for AFLP and MSAP analyses.

Table S2 Measures of genetic and epigenetic diversity in *Plantago lanceolata*.

Table S3 AMOVA results.

Table S4 (a) Regressions comparing epigenetic variation of parent and offspring populations. (b) Locus-by-locus transmissibility.

Table S5 Correlation analyses between epigenetic and genetic diversity, and between epigenetic and phenotypic diversity.

Table S6 Results of GLM analyses of land-use effects on epigenetic and genetic diversity.

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New Phytologist **Supporting Information**

Article title:

Structure, stability and ecological significance of natural epigenetic variation: a large-scale survey in *Plantago lanceolata*

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The following Supporting Information is available for this article:

Table S1 Adaptor- and primer sequences used for AFLP and MSAP analyses.

Primer	Sequence
Adaptors	
*EcoR*I-*adapter* top	5'-CTCGTAGACTGCGTACC-3'
*EcoR*I-*adapter* bottom	5'-AATTGGTACGCAGTCTAC-3'
*Mse*I-*adapter* top (AFLP)	5'-GAGCGATGAGTCCTGAG-3'
*Mse*I-*adapter* bottom (AFLP)	3'-TACTCAGGACTCAT-5'
*Hpa*II/*Msp*I-*adapter* top (MSAP)	5'-GATCATGAGTCCTGCT-3'
*Hpa*II/*Msp*I -adapter bottom (MSAP)	5'-CGAGCAGGACTCATGA-3'
Preselective primers	
*Eco*RI + A	5'-GACTGCGTACCAATTCA-3'
*Mse*I + C (AFLP)	5' GATGAGTCCTGAGTAAC-3'
*Hpa*II/*Msp*I (MSAP)	5'-ATCATGAGTCCTGCTCGG-3'
Selective primer AFLP	
*Eco*RI + AAC-FAM[1]	5'-GACTGCGTACCAATTCAAC-3'
*Eco*RI + ACA-VIC[2]	5'-GACTGCGTACCAATTCACA-3'
*Eco*RI + ACC-NED[3]	5'-GACTGCGTACCAATTCACC -3'
*Eco*RI + AGC-PET[4]	5'-GACTGCGTACCAATTCAGC-3'
*Mse*I + CTA[1,2,3]	5' GATGAGTCCTGAGTAACTA-3'
*Mse*I + CAG[4]	5' GATGAGTCCTGAGTAACAG-3'
Selective primers MSAP	
*Eco*RI + AAC-FAM[1,2,3]	5'-GACTGCGTACCAATTCAAC-3'
*Eco*RI + ACT- FAM[4]	5'-GACTGCGTACCAATTCACT-3'
*Eco*RI + ACA-VIC[5,6]	5'-GACTGCGTACCAATTCACA-3'
*Eco*RI + ACG-VIC[7,8]	5'-GACTGCGTACCAATTCACG-3'
*Hpa*II/*Msp*I + CAA[1,4,7]	5'-ATCATGAGTCCTGCTCGGTCAA-3'
*Hpa*II/*Msp*I + CAG[5]	5'-ATCATGAGTCCTGCTCGGTCAG-3'
*Hpa*II/*Msp*I + CAT[2,6]	5'-ATCATGAGTCCTGCTCGGTCAT-3'
*Hpa*II/*Msp*I + CGA[8]	5'-ATCATGAGTCCTGCTCGGTCGA-3'
*Hpa*II/*Msp*I + CTA[3]	5'-ATCATGAGTCCTGCTCGGTCTA-3'

Superscript numbers indicate primer combinations used for the selective amplification

Table S2 Measures of genetic and epigenetic diversity in Plantago lanceolata. Number of loci: AFLP=545, MASP-n=560, MSAP-m=430, MSAP-h=491

		Shannon diversity (*H'*)								Number of polymorphic loci								Number of private loci							
		AFLP		MSAP-n		MSAP-m		MSAP-h		AFLP		MSAP-n		MSAP-m		MSAP-h		AFLP		MSAP-n		MSAP-m		MSAP-h	
		F	GH	F	GH	F	GH	F	GH	F	GH	F	GH	F	GH	F	GH	F	GH	F	GH	F	GH	F	GH
Alb	max	0.28	0.28	0.24	0.23	0.16	0.15	0.14	0.11	165	168	146	139	105	98	75	58	2	1	3	4	6	5	4	7
	mean	0.25	0.25	0.20	0.19	0.14	0.13	0.11	0.09	147	148	123	118	93	91	60	50	0.53	0.19	1.00	1.13	1.63	2.00	1.21	1.63
	min	0.22	0.20	0.18	0.17	0.10	0.11	0.09	0.07	133	119	109	104	76	83	49	36	0	0	0	0	0	0	0	0
Hainich	max	0.29	0.32	0.25	0.23	0.16	0.17	0.15	0.13	175	189	152	140	99	105	81	71	2	1	2	3	3	6	4	7
	mean	0.25	0.26	0.21	0.20	0.14	0.14	0.13	0.11	148	157	127	122	89	92	67	57	0.46	0.25	0.69	1.05	1.38	1.75	2.08	1.95
	min	0.20	0.23	0.18	0.17	0.12	0.10	0.10	0.08	120	135	111	103	77	77	54	44	0	0	0	0	0	0	0	0
Schorfheide	max	0.29	0.32	0.24	0.22	0.18	0.17	0.16	0.11	172	188	147	133	110	102	84	59	2	2	4	2	5	6	4	4
	mean	0.26	0.27	0.22	0.20	0.15	0.14	0.13	0.09	154	160	132	120	91	94	70	51	0.60	0.44	1.20	0.83	1.93	1.22	1.93	1.50
	min	0.23	0.23	0.16	0.17	0.12	0.12	0.10	0.08	135	137	99	101	79	86	51	43	0	0	0	0	0	0	0	0
Overall	max	0.29	0.32	0.25	0.23	0.18	0.17	0.16	0.13	175	189	152	140	110	105	84	71	2	2	4	4	6	6	4	7
	mean	0.25	0.26	0.21	0.20	0.14	0.14	0.12	0.10	149	155	127	120	91	92	65	53	0.53	0.30	0.98	1.00	1.66	1.65	1.68	1.70
	min	0.20	0.20	0.16	0.17	0.10	0.10	0.09	0.07	120	119	99	101	76	77	49	36	0	0	0	0	0	0	0	0

F: field populations; GH: glasshouse populations; MSAP-n, -m, -h: non-methylated, methylated and hemimethylated subepiloci, respectively; N: number of analysed loci.

Table S3 AMOVA results. P-values lower than 0.05 marked in bold and highlighted in grey.

		AFLP			MSAP-n			MSAP-m			MSAP-h		
		Among		Within	Among		Within	Among		Within	Among		Within
	statistic	regions	pops within regions	pops	regions	pops within regions	pops	regions	pops within regions	pops	regions	pops within regions	pops
Field	σ	1.31	0.89	49.58	0.93	0.97	42.45	0.39	0.32	21.96	0.32	0.32	21.62
	Var %	2.53	1.72	95.75	2.09	2.18	95.73	1.73	1.42	96.85	1.42	1.42	97.16
	P	**0.001**	**0.002**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.029**	**0.001**	**0.001**	**0.011**	**0.001**
Glasshouse	σ	0.63	-0.42	51.65	0.62	0.63	39.98	0.20	0.06	21.17	0.09	0.36	17.51
	Var %	1.22	-0.81	99.59	1.49	1.53	96.97	0.96	0.27	98.78	0.51	2.00	97.49
	P	**0.001**	0.929	0.508	**0.001**	**0.003**	**0.001**	**0.001**	0.328	0.092	**0.001**	**0.003**	**0.001**

		Among			Among			Among			Among		
		regions	pops within regions	all pops	regions	pops within regions	all pops	regions	pops within regions	all pops	regions	pops within regions	all pops
Field	Φ	0.025	0.018	0.043	0.021	0.022	0.043	0.017	0.014	0.032	0.014	0.014	0.028
Glasshouse		0.012	-0.008	0.004	0.015	0.016	0.030	0.010	0.003	0.012	0.005	0.020	0.025

Table S4a Regressions comparing epigenetic variation of parent and offspring populations. P-values lower than 0.05 marked in bold and highlighted in grey.

		AFLP		MSAP-n		MSAP-m		MSAP-h	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Shannon's H'	parent	1.21	0.278	9.03	**0.005**	8.18	**0.007**	7.35	**0.010**
	region	3.14	0.055	0.48	0.621	3.82	**0.031**	14.80	**0.000**
	parent:region	1.82	0.175	0.31	0.736	1.08	0.350	0.63	0.539
Nei's distance	parent	40.29	**0.000**	69.02	**0.000**	50.56	**0.000**	53.87	**0.000**
	region	11.19	**0.000**	13.40	**0.000**	4.63	**0.014**	12.72	**0.000**
	parent:region	1.25	0.297	0.27	0.763	0.01	0.987	0.83	0.444

Table S4b Locus-by-locus transmissibility. In AFLP, considering the shared absences is meaningful, as they do represent an allele; in case of MSAP, however, they are not informative. Values considered afterwards are in bold.

Shared absences	AFLP	MSAP-n	MSAP-m	MSAP-h
Yes	**85.87%**	89.33%	91.73%	93.07%
No	67.23%	**56.94%**	**51.79%**	**40.09%**

Table S5 Correlation test results between epigenetic and genetic diversity, and between epigenetic and phenotypic diversity.

	FIELD						GLASSHOUSE					
	MSAP-n		MSAP-m		MSAP-h		MSAP-n		MSAP-m		MSAP-h	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Genetic diversity	0.18	0.23	0.11	0.46	0.29	0.05	0.14	0.329	0.220	0.118	0.160	0.260
Phenotypic diversity	0.14	0.37	-0.03	0.87	0.18	0.25	-0.02	0.867	0.11	0.415	-0.09	0.498

Table S6 Results of GLM analyses of land use effects on epigenetic and genetic diversity. P-values lower than 0.05 marked in bold and highlighted in grey.

Marker	AFLP		MSAP-n		MSAP-m		MSAP-h	
Source	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Mowing	0.58	0.448	0.62	0.432	0.51	0.478	5.20	**0.025**
Fertilisation	0.25	0.620	0.17	0.679	0.02	0.892	0.81	0.371
Grazing	3.53	0.064	2.93	0.091	11.59	**0.001**	0.48	0.490
Region	3.35	**0.040**	1.82	0.169	2.39	0.099	4.72	**0.012**
Growing Environment (GE)	4.52	**0.037**	13.34	**0.000**	3.65	0.060	92.20	**0.000**
Mowing * Region	2.08	0.132	5.98	**0.004**	1.25	0.294	0.16	0.854
Fertilisation * Region	0.98	0.382	1.67	0.195	0.91	0.408	1.28	0.284
Grazing * Region	0.85	0.431	0.61	0.548	0.69	0.504	1.63	0.203
Mowing * GE	0.04	0.848	1.42	0.237	0.19	0.666	1.41	0.238
Fertilisation * GE	0.65	0.422	2.89	0.093	0.20	0.658	0.94	0.336
Grazing * GE	2.58	0.112	0.47	0.494	0.01	0.939	7.35	**0.008**
Region * GE	0.67	0.516	0.20	0.820	0.34	0.714	2.20	0.118
Mowing * Region * GE	0.28	0.760	1.24	0.295	0.46	0.632	1.09	0.341
Fertilisation * Region * GE	0.37	0.691	0.73	0.483	0.38	0.686	0.43	0.655
Grazing * Region * GE	0.37	0.695	1.30	0.278	1.03	0.361	1.66	0.197
Model R^2	0.28		0.39		0.28		0.63	

Growing environment: field or glasshouse

Methods S1

AFLP and MSAP protocol, adapted from Schultz et al. (2014).

For restriction and ligation (RL) 5.2 µl genomic DNA were combined with 5.8 µl RL reaction mix containing 0.55 µl BSA (1 mg/ml; New England Biolabs, NEB), 1.1 µl 0.5 M NaCl, 5 u *Eco*RI (NEB), 1 u *Mse*I (NEB), 67 u T4 DNA ligase (NEB), 1.1 µl T4 DNA ligase buffer (NEB), 1 µl *Eco*RI adapter (5 pmol) and 1 µl *Mse*I adapter (50 pmol). The reaction was incubated for 2 h at 37 °C and diluted 1:5. For the preselective amplification (PCR1), 4 µl RL product were combined with 16 µl PCR1 reaction mix containing 1.5 ng/µl *Eco*RI- and *Mse*I preselective primers each, 200 µM dNTPs (Roth), 2 µl 10 x Dream Tag buffer (QIAGEN), 0.8 u Dream Taq polymerase (QIAGEN) and 9.84 µl H_2O. The thermocycler protocol was 72.0°C (2 min) followed by 20 cycles of 94.0°C (20 s), 56.0°C (30 s) and 72.0°C (2 min) and a final extension at 60.0°C (30 min), performed on an Eppendorf Mastercycler gradient. The PCR1 product was diluted 1:5. For the selective amplification (PCR2), 2.2 µl PCR1 product was combined with 7.8 µl PCR2 reaction mix containing 5 µl Multiplex PCR kit (QIAGEN) and 1.4 µl fluorescent labeled *Eco*RI primer (1 pmol/µl) and 1.4 µl *Mse*I (5 pmol/µl) selective primers each. The thermocycler protocol was 94.0°C (2 min) followed by 10 cycles of 94.0°C (20 s), 66.0°C (30 s, decreasing 1°C per cycle) and 72.0°C (2 min) and 20 cycles of 94.0°C (20 s), 56.0°C (30 s) and 72.0°C (2 min), and a final extension at 60.0°C (30 min), performed on an Eppendorf Mastercycler pro 384.
The MSAP protocol was almost identical with the AFLP protocol, except exchanging the restriction enzyme *Mse*I by 5 u *Hpa*II or *Msp*I (Fermentas) and replacing the *Mse*I adaptor and primers by the respective *Hpa*II/*Msp*I adaptor and primers in equal concentrations.

After an initial screening of 32 primer pairs, eight selective primer combinations (Supplementary Table S1) were chosen for MSAP analyses. Separation and visualization of the fragments was done on a ABI 3130 capillary sequencer (Applied Biosystems, Foster City, USA) with Genescan 500(-250) LIZ internal size standard (Applied Biosystems). GENMAPPER version 5.0 (Applied Biosystems) was used to analyze the AFLP profiles. Binning of fragments was done manually for all samples in one batch using a peak height threshold of 10 rfu. Peak height data were exported and for each fragment a specific peak height threshold was manually determined based

on the peak height distribution which allowed scoring presence (1) and absence (0) of fragments. All loci that showed a monomorphic pattern or a deviation in only one individual were excluded from the data set to prevent biased parameter estimation. Error rate estimation was based on 40 replicate samples (12%) that were repeated, starting from the same DNA extracts.

Reference:

Schulz B, Eckstein RL, Durka W. **2014**. Epigenetic variation reflects dynamic habitat conditions in a rare floodplain herb. *Molecular Ecology* **23**: 3523–3537.

Chapter III

Rapid evolution in managed grasslands: different land-use regimes are associated with contrasting phenotypes in *Plantago lanceolata*

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Abstract

Land-use intensification is a major driver of biodiversity declines, and it is known to decrease species numbers and alter community composition of managed grasslands. An open question is whether similar impacts occur within species, i.e. whether grassland management affects also the genetic diversity of plant populations and alters their genetic composition, possibly reflecting adaptation to land use. To address these questions, we sampled 61 populations of the common grassland herb *Plantago lanceolata* that covered a broad range of land use intensities in the German Biodiversity Exploratories, and we grew their offspring in a common environment to quantify variation in plant size, architecture, reproduction, and leaf economy. All measured traits harboured substantial heritable variation, and six out of nine traits showed population differentiation. Interestingly, several traits were significantly correlated with land use intensity, but with opposing trends for mowing versus grazing: Increased mowing intensity was associated with larger plant size and lower specific leaf area (SLA), which may reflect evolutionary responses to increased light competition and a lesser need for resource conservation in highly productive meadows. In contrast, increased grazing intensity tended to be associated with smaller plant size and higher SLA, a phenotype syndrome known from grazing lawns. Moreover, we found that land-use intensification also affected genetic diversity, again with opposing effects for mowing versus grazing: while increased mowing was associated with decreased levels of intrapopulation phenotypic variation, the opposite was true for increased grazing intensity. In summary, land use intensification has not only already caused rapid evolutionary changes in these grassland populations, it also affects their future evolutionary potential.

Key words: fertilisation, genetic differentiation, grassland management, grazing, mowing, plant size, rapid evolution, specific leaf area.

Introduction

Human activity is radically changing the global environment, and it is causing substantial biodiversity declines in all biomes. Recent estimates suggest that species richness has globally decreased by an average of 13.6% since the 16th century, with the greatest declines in the 19th and 20th centuries, and a predicted further 3.4% loss until the end of this century (Newbold *et al.* 2015). Among the many factors contributing to these biodiversity losses, the conversion of natural ecosystems to managed land and the intensification of existing land use practices are among the strongest ones (Sala *et al.* 2000; Foley *et al.* 2005).

While natural biodiversity is impacted by humans, the mechanisms of evolutionary change never cease, new diversity continuously emerges through mutation, and existing diversity is modulated through genetic drift, migration and natural selection. Plant species with broad environmental ranges can be under divergent selection in different environments, and indeed, local adaptation has been widely documented, especially in species with larger populations (Leimu & Fischer 2008). These contrasting environments need not necessarily be located far from each other; in heterogeneous environments divergent selection and local adaptation can take place at very small scales (Linhart & Grant 1996), as it has been shown for example in heavy metal-contaminated soils (Antonovics 1971; Jiménez-Ambriz *et al.* 2007), or even for small-scale herbivore abundances (Dirzo & Harper 1982). Another type of biotic interaction that can create such heterogeneous mosaics are land-use practices in anthropogenic agricultural landscapes, which might strongly differ between neighbouring pieces of land.

Subsistence pastoral systems and the grazing of grasslands by livestock dates back to over 11.000 years, while mowing of meadows for haymaking – a prerequisite for feeding domesticated animals year-round – can be traced back to around 1000 BC (Briggs 2009). Both land-use practices strongly affect the composition of plant communities, however in different ways. Mowing generally creates a uniform, low-canopy, high-light environment, while grazing is heterogeneous on various scales through the selective removal of biomass, and by trampling and depositing dung and urine in patches (Gibson 2009). Researchers repeatedly documented evolutionary

responses to different grassland management regimes, starting as early as the 1920's (Stapledon 1928), in classical work from English grasslands and the Park Grass Experiment (e.g. Warwick & Briggs 1979; Davies & Snaydon 1976; Silvertown *et al.* 2006), and in more recent and contemporary studies (e.g. Suzuki 2008). However, most previous studies included few populations and coarse categories of land-use types, with some exceptions such as two recent studies from the German Biodiversity Exploratories (Völler *et al.* 2013; 2017).

The Biodiversity Exploratories (www.biodiversity-exploratories.de) are a long-term research platform designed for studying the relationships between land use, biodiversity and ecosystem functioning. They provide networks of large numbers of standardised grassland and forest plots across three regions in the north, middle and south of Germany (Fischer *et al.* 2010). In the grassland plots, the intensities of mowing, fertilisation and grazing are precisely documented for each plot through annual inventories, providing unique opportunities for studying many populations of the same species along broad gradients of land-use intensities (Blüthgen *et al.* 2012).

Previous studies of land use effects on species diversity often found that species richness of plants decreases with increasing land use intensity, as less competitive species are excluded by dominant competitors at higher nutrient availability (Blüthgen *et al.* 2012). It is still unclear, however, whether similar processes take place at the intraspecific level (Vellend & Geber 2005). In the Biodiversity Exploratories, intraspecific trait variation of grassland plants has been recently studied through a phytometer approach (Herz *et al.* 2017), where multiple species were planted into different plots to demonstrate that changes in land use intensity can create substantial variation in phenotypic traits. However, while this study controlled for plant genotypes to estimate only the plastic components of intraspecific variation, the opposite approach is required if we want to understand adaptation and evolutionary potential: a common-garden approach where genotypes from different origins are planted under controlled environmental conditions, so that only the heritable component of intraspecific variation is remaining. This is the approach we chose for our study with *Plantago lanceolata*.

We chose to work with *Plantago lanceolata*, because it is one of the most widespread grassland species in the Biodiversity Exploratories. Although there has been previous work on *P. lanceolata* in the context of intraspecific variation and land use (Kuiper and Bos 1992), this work was usually restricted to few populations and coarse comparisons of land use types (Warwick & Briggs 1979; van Groenendael 1986; Wolff & Van Delden 1987; van Tienderen & van der Toorn 1991). Here, we studied heritable phenotypic variation in 61 grassland populations of *P. lanceolata* from a broad range of (precisely known) intensities of mowing, fertilisation and grazing. We asked the following questions: (i) Do the studied populations harbour significant heritable intraspecific variation? (ii) If yes, how are the mean phenotypes related to land use intensity? (iii) Does land-use intensification also affect the within-population diversity of phenotypic traits?

Materials and Methods

Plant material and common-garden experiment

Plantago lanceolata L. (Plantaginaceae) is a very common Eurasian grassland rosette herb, distributed over broad geographic and environmental gradients. It is also among the commonest plant species in the Biodiversity Exploratories, occurring on 70% of the grassland plots. The sampling design and experimental setup are depicted and described in detail in Gáspár *et al.* (2019). Briefly, in September 2015, we collected ripe seeds from altogether 61 plots across the three regions of the Biodiversity Exploratories. We used these seeds to establish a common-garden experiment in a greenhouse at the University of Tübingen. The experiment had a randomised block design with three seedlings from each of four mother plants (= four maternal seed families) per sampled plot (total $N = 741$). It was maintained under a 16 h/8 h day/night cycle at 21°C/15°C. After 10 weeks, we harvested all plants, scanned five leaves per plant for further analyses, and dried all plant material at 70°C for at least 72 h. To quantify intraspecific variation in plant size, architecture, reproduction and leaf economy, we measured the following phenotypic traits: (1) aboveground biomass, (2) length of the longest leaf, (3) plant height, (4) the shape (width:length ratio) of leaves and (5) their specific leaf area (SLA; area of five leaves

divided by their dry weight), (6) the 'growth habit' of the plants (rosette height:width ratio), (7) onset of flowering, (8) number of inflorescences, and (9) reproductive allocation (reproductive:total aboveground biomass ratio).

Data analysis

We used the R statistical environment (R Development Core Team 2008) for all statistical analyses. Prior to the main analyses, we z-transformed (standardised) all nine response variables by subtracting the mean and dividing by the standard deviation. To simplify our subsequent analyses, and to account for all differences between regions that were of no interest to our study (e.g. altitude, latitude, etc.), we then fitted a linear model for each variable with regions of origin and experimental blocks as main effects, and we used the residuals from these models in all analyses described below (Manning *et al.* 2015; Soliveres *et al.* 2016). To explore overall phenotypic similarities and visualise our data, we ran a principal component analyses (PCAs) on the maternal seed family-level aggregated data using the *vegan* package (Oksanen *et al.* 2017). Next, we tested for heritable variation in phenotypes with two approaches: First, we used the *lme4* package (Bates *et al.* 2015) to fit mixed-effects models that included populations of origin as fixed effect and maternal seed families as random effect. In these models, any significant effect indicates resemblance among relatives and thus heritable variation in a trait. In a second approach, we calculated narrow-sense heritabilities for each trait and population as $h^2 = V_A / (V_A + V_\varepsilon) = 4V_{FAM} / (4V_{FAM} + V_\varepsilon)$, where V_A is the additive genetic variance that is equal to four times the variance among families (V_{FAM}) in half-sib experimental setups, and V_ε is the residual variance (Petit *et al.* 2001). However, the obtained h^2 values showed a zero-inflated distribution (data not shown), probably because the data were based on only three individuals per family and four families per population – an inevitable consequence of our aim to maximise the number of populations while keeping the total number of samples at a manageable level. As an alternative, we also calculated the phenotypic diversity for each trait and population as the standard deviation of the family-level means within a population. h^2 and phenotypic diversity turned out to be strongly correlated in all traits ($r = 0.323$ to 0.751, all $P < 0.001$), and

we therefore used the phenotypic diversity in all further analyses. Finally, to explore relationships between land use intensity and phenotypic traits, we added the vectors of mowing, fertilisation and grazing to the PCAs with the *envfit* function in the *vegan* package, and we related population-level trait means (first aggregated at the family level) or phenotypic diversities, respectively, to mowing, fertilisation and grazing intensity by running separate linear models for each trait and land use component. We corrected all *P*-values for false discovery rates (FDR). The land-use intensity data comes from yearly inventories in the Biodiversity Exploratories, and is based on the number of mowing events, the amount of nitrogen added per hectare, and the number of grazing animals (livestock units) per hectare in a year (for more details see Blüthgen *et al.* 2012).

Results

The mixed-effect models showed significant population differentiation in six out of the nine studied phenotypic traits, and there were significant maternal seed family effects – indicating heritable variation within populations – in all of the measured traits (Table 1). Across traits, an average of 6.4% of the variance (range 2–11%) resided among populations, and 14.6% (range 11–26%) among families within populations. The first two axes of the principal component analysis explained 55.3% of the multi-trait phenotypic variance and were associated with different traits (Figure 1 and Table 1). Moreover, the PCA indicated three groups of closely related traits: (1) the reproduction-related traits of flowering time, number of inflorescences and reproductive allocation, (2) aboveground biomass and SLA, which were negatively correlated, and (3) the remaining traits, all related to plant architecture and leaf shape.

When we related population-level phenotypic variation to land use intensity, we found the strongest patterns for mowing intensity: at higher mowing intensities, plants were taller, had longer leaves and a greater biomass, but a lower specific leaf area (Table 2). There were also positive relationships between fertilisation and biomass, and between grazing intensity and specific leaf area, but these did not remain statistically significant after correcting for false discovery rates (Table 2). An

intriguing general pattern was that the signs of relationships tended to be opposite for grazing versus mowing and fertilisation (Table 2). For instance, high population-level values of specific leaf area were associated with low levels of mowing intensity but high levels of grazing intensity (Fig. 2). The pattern was further supported by the PCA, where the vectors for mowing and fertilisation versus grazing had opposite directions (Fig. 1), i.e. they were associated with contrasting multi-trait phenotypes.

There were also several patterns of relationships between land use intensities and within-population trait diversities, particularly for mowing intensity (Table 3). Although none of these remained statistically significant after FDR correction, there was again an overall pattern that effects were in opposite directions for grazing versus mowing and fertilisation. While higher intensities of mowing and fertilisation were generally associated with lower phenotypic diversity, the opposite was true for increasing grazing intensity (Table 3, Fig. 3).

Discussion

Land use intensification and its ecological and evolutionary consequences are important research topics in current evolutionary ecology. Here, we investigated whether the common grassland plant *Plantago lanceolata* is undergoing evolutionary changes in response to land-use intensification. We found substantial heritable phenotypic variation – indicating evolutionary potential – in all studied traits, and that there were some significant associations between phenotypes and land use intensity. Most interestingly, throughout our analyses, mowing and fertilisation tended to be associated with contrasting phenotypes, and they also had opposing effects on within-population phenotypic diversity, suggesting that these two land use processes have fundamentally different effects on the evolution of these plant populations.

Several previous studies already studied intraspecific variation of *P. lanceolata* (e.g. Sagar & Harper 1964; Warwick & Briggs 1979; Kuiper & Bos 1992), or relationships between intraspecific variation and environment of origin. One classic study showed substantial population differentiation as well as within-population variation in five reproductive traits among eight natural *P. lanceolata* populations

(Primack & Antonovics 1981). Other studies found strong heritable differences between plants from two closely located contrasting habitats (van Groenendael 1986), and even local adaptation with consistent better survival at the home site (Van Tienderen & van der Toorn 1991). Another previous study found substantially higher population differentiation in four of the traits we also measured (Wolff & Van Delden 1987). However, some of this discrepancy may result from differences in experimental design, since Wolff & Van Delden (1987) studied F_2 plants from full-sib families (from only two pastures and two meadows), whereas we worked with F_1 individuals from half-sib families that – as a result of wind pollination and obligate outcrossing – still contained random paternal alleles, and therefore harboured greater within-population variance. In summary, while estimates of phenotypic variation tended to be lower in our study than in some previous studies on *P. lanceolata*, our results confirmed the existence of significant, and potentially adaptive, heritable variation in phenotype.

Land use and population-level phenotypic trait means

When examining patterns of phenotypic variation in relation to land use intensity, the most intriguing result were the contrasting effects with regard to grazing and mowing. Plants originating from sites with high mowing intensity were in general larger (in terms of aboveground biomass, length of the longest leaf and height of the longest inflorescence stalk) and they had a lower specific leaf area, whereas plants from sites with high grazing intensity tended to be smaller, with higher SLA. These opposite effects of the two land use types consistently appeared across almost all of the measured traits. Trait relationships with fertilisation intensity followed mostly the same direction as mowing; it is well known from the Biodiversity Exploratories that these two land-use components are strongly correlated (Blüthgen *et al.* 2012). There are some previous studies of *P. lanceolata* comparing different land use types (van Groenendael 1986; Wolff & Van Delden 1989; Kuiper & Bos 1992) which also found that a prostrate growth habit with more dormant seeds is associated with grazing, whereas in meadows there are taller and larger plants with more, readily germinating seeds. However, these studies compared only a handful of sites with discrete

categories of land use, often confounded with other environmental factors (e.g. grazed sand dunes vs. wet, nutrient-rich hayfields). Our study corroborates these findings across a much larger number of natural populations and broad gradients of mowing and grazing intensities. Similar to ours, other studies from the Biodiversity Exploratories showed that patterns of phenotypic trait differentiation were strongest in relation to mowing (Völler *et al.* 2013; Völler *et al.* 2017).

We found that lower values of specific leaf area were associated with larger plants and more intense mowing, but that SLA increased with decreasing plant size and increasing grazing. At first glance this result is surprising because higher SLA is in generally thought to be associated with higher relative growth rate, leaf nitrogen content and shading (Lambers & Poorter 1992). However, most of these relationships have been observed across species, often with a strong emphasis on woody species (Wright *et al.* 2004; Poorter *et al.* 2009; Díaz *et al.* 2016). Several previous studies quantified intraspecific variation in SLA but only the plastic components of it, or were unable to separate plastic and heritable components, e.g. where intraspecific variation was measured in different habitats in the field (e.g. Shipley & Almeida-Cortez 2003; Bilton *et al.* 2010; Hulshof *et al.* 2013; Jung *et al.* 2014). We are aware of only one other study explicitly focusing on heritable intraspecific variation in SLA in a grassland plant: Scheepens *et al.* (2010) conducted a reciprocal transplant experiment with *Campanula thyrsoides* across different altitudes, and they found that there was significant heritable differentiation in SLA among populations.

Some previous studies might help to explain the observed relationships between SLA, plant size and land use. A multi-species study across 157 species showed that in the majority of these, including 14 out of 20 studied herbaceous species, SLA decreased with increasing leaf area (Milla & Reich 2007). The authors explained this by the increased need for structural support in larger plants. In the Biodiversity Exploratories, more intensely mown plots are usually also more fertilised (Blüthgen *et al.* 2012), with more intense competition for light (Kuiper & Bos 1992, page 273), resulting in larger and taller plants that must invest more in structural support, thereby decreasing SLA. On the mown plots, nutrients are readily supplied

by fertilisation, so there is no selection for resource conservation, but the race for light – following each mowing that "cleans the slate" homogeneously across the whole plot – might select for taller plants with bigger and stronger leaves. Another reason why SLA of *P. lanceolata* is positively related to grazing could be that the trait is functionally related to herbivory. Plants with high SLA are often not only shorter-lived but also more palatable (Reich *et al.* 1999; Poorter *et al.* 2009). If these plants have a high tolerance of grazing, i.e. they regrow better than competitors or even overcompensate, then grazing will select for high SLA. In a multispecies study from southern Patagonia, Cingolani *et al.* (2005) indeed showed that a positive feedback loop can increase the abundance of preferentially eaten plants. They argued that this generally requires three conditions to be met: high levels of grazing tolerance, herbivore preference, and high resource availability. *P. lanceolata* is known to be grazing-tolerant but preferentially grazed by cattle and sheep (Sagar & Harper 1964). It is therefore possible that this explanation is relevant for our study system, and grazing selects for small but rapidly regrowing genotypes with high SLA.

We did not find land-use related population differentiation in growth habit (related to leaf angle), as it was the case in earlier studies of *Plantago lanceolata* (van Groenendael 1986; Wolff & Van Delden 1989). One explanation for this could be that our F_1 plant material with random fathers had too high levels of within-family variability, compared to several generations of selective breeding in a previous experiment that found strong heritability of this trait (Wolff & Van Delden 1989). Another explanation might be that, unlike previous studies with simple comparisons of land-use categories, we analysed gradients of land-use intensity, and relationships between leaf angle and land use might be complex or non-linear.

Genetic diversity in phenotypic traits

All phenotypic traits analysed in our study showed significant variation at the level of seed families within populations, which allowed us to examine relationships between within-population phenotypic diversity and land use. We found that mowing generally decreased phenotypic diversity, while grazing tended to increase it. The most likely explanation for these contrasting results is that mowing and fertilisation generally

homogenise environmental conditions across a meadow, whereas grazing creates heterogeneity and a greater diversity of microhabitats (Bakker *et al.* 1984). Although the extent of the heterogenising effect of grazing depends on the quality of grazing (e.g. livestock density and selective browsing of different livestock species), the spatial structure of vegetation, and the scale of study (Adler *et al.* 2001), pastures as the ones studied here typically experience more heterogeneous biomass removal, nutrient supply, disturbance, and competition than similar, but mown, grasslands (Bakker *et al.* 1983; Bakker *et al.* 1984; McNaughton 1984). Further evidence for this comes from a recent study in the Biodiversity Exploratories that found land use, especially mowing intensification, to cause biotic homogenisation across 12 trophic groups (Gossner *et al.* 2016). In summary, we found that increased mowing decreased within-population phenotypic diversity in *Plantago lanceolata* populations, but that the opposite was true for increased grazing, and we suggest that these contrasting results are explained by the heterogenising qualities of grazing versus homogenising effects of mowing and fertilisation.

Conclusions

Our study demonstrates that increased mowing and grazing affects evolution and diversity of phenotypes in a common grassland plant. On the one hand, different land use practices were associated with contrasting multi-trait phenotypes, most likely reflecting adaptation to the selection regimes exerted by these land use practices. On the other hand, mowing and grazing had opposing effects on within-population diversity, presumably because mowing makes habitat conditions more homogenous whereas the opposite is true for grazing. Taken together, land use intensification not only causes rapid evolution of phenotypes in the studied grassland populations, but it also affects their future evolutionary potential.

Authors' contributions

OB and WD planned and designed the research; BG conducted fieldwork and performed experiments; BG and OB analysed the data and wrote the manuscript with input of WD.

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References

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

Antonovics J. 1971. The effects of a heterogeneous environment on the genetics of natural populations: The realization that environments differ has had a profound effect on our views of the origin and role of genetic variability in populations. *American Scientist* 59: 593–599.

Bakker JP, De Bie S, Dallinga JH, Tjaden P, De Vries Y. 1983. Sheep-grazing as a management tool for heathland conservation and regeneration in the netherlands. *The Journal of Applied Ecology* 20: 541–560.

Bakker JP, de Leeuw J, van Wieren SE. 1984. Micro-patterns in grassland vegetation created and sustained by sheep-grazing. *Vegetatio* 55: 153–161.

Bates D, Mächler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67: 1–48.

Bilton MC, Whitlock R, Grime JP, Marion G, Pakeman RJ. 2010. Intraspecific trait variation in grassland plant species reveals fine-scale strategy trade-offs and size differentiation that underpins performance in ecological communities. *Botany* 88: 939–952.

Birkhofer K, Schöning I, Alt F, Herold N, Klarner B, Maraun M, Marhan S, Oelmann Y, Wubet T, Yurkov A, *et al.* 2012. General relationships between abiotic soil properties and soil biota across spatial scales and different land-use types. *PloS One* 7: e43292.

Blüthgen N, Dormann CF, Prati D, Klaus VH, Kleinebecker T, Hölzel N, Alt F, Boch S, Gockel S, Hemp A, *et al.* 2012. A quantitative index of land-use intensity in grasslands: Integrating mowing, grazing and fertilization. *Basic and Applied Ecology* 13: 207–220.

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

Cingolani AM, Posse G, Collantes MB. 2005. Plant functional traits, herbivore selectivity and response to sheep grazing in Patagonian steppe grasslands. *The Journal of Applied Ecology* 42: 50–59.

Davies M, Snaydon R. 1976. Rapid population differentiation in a mosaic environment. III. Measures of selection pressures. *Heredity* 36: 59–66.

Des Roches S, Post DM, Turley NE, Bailey JK, Hendry AP, Kinnison MT, Schweitzer JA, Palkovacs EP. 2018. The ecological importance of intraspecific variation. *Nature Ecology & Evolution* 2: 57–64.

Díaz S, Kattge J, Cornelissen JHC, Wright IJ, Lavorel S, Dray S, Reu B, Kleyer M, Wirth C, Prentice IC, *et al.* 2016. The global spectrum of plant form and function. *Nature* 529: 167–171.

Dirzo R, Harper JL. 1982. Experimental studies on slug-plant interactions: III. Differences in the acceptability of individual plants of *Trifolium repens* to slugs and snails. *The Journal of Ecology*: 101–117.

Fischer M, Bossdorf O, Gockel S, Hänsel F, Hemp A, Hessenmöller D, Korte G, Nieschulze J, Pfeiffer S, Prati D, *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, Barford C, Bonan G, Carpenter SR, Chapin FS, Coe MT, Daily GC, Gibbs HK, *et al.* 2005. Global consequences of land use. *Science* 309: 570–574.

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

Gibson DJ. 2009. *Grasses and Grassland Ecology*. Oxford University Press.

Gossner MM, Lewinsohn TM, Kahl T, Grassein F, Boch S, Prati D, Birkhofer K, Renner SC, Sikorski J, Wubet T, *et al.* 2016. Land-use intensification causes multitrophic homogenization of grassland communities. *Nature* 540: 266–269.

van Groenendael JM. 1986. Life history characteristics of two ecotypes of *Plantago lanceolata* L. *Acta Botanica Neerlandica* 35: 71–86.

Herz K, Dietz S, Haider S, Jandt U, Scheel D, Bruelheide H. 2017. Drivers of intraspecific trait variation of grass and forb species in German meadows and pastures. *Journal of Vegetation Science* 28: 705–716.

Hulshof CM, Violle C, Spasojevic MJ, McGill B, Damschen E, Harrison S, Enquist BJ. 2013. Intra-specific and inter-specific variation in specific leaf area reveal the importance of abiotic and biotic drivers of species diversity across elevation and latitude. *Journal of Vegetation Science* 24: 921–931.

Jiménez-Ambriz G, Petit C, Bourrié I, Dubois S, Olivieri I, Ronce O. 2007. Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *The New Phytologist* 173: 199–215.

Kuiper PJC, Bos M. 1992. *Plantago: A Multidisciplinary Study*. Springer.

Lambers H, Poorter H. 1992. Inherent Variation in Growth Rate Between Higher Plants: A Search for Physiological Causes and Ecological Consequences. In: Begon M, Fitter AH, eds. *Advances in Ecological Research*. Academic Press, 187–261.

Leimu R, Fischer M. 2008. A meta-analysis of local adaptation in plants. *PloS One* 3: e4010.

Linhart YB, Grant MC. 1996. Evolutionary significance of local genetic differentiation in plants. *Annual Review of Ecology and Systematics* 27: 237–277.

Manning P, Gossner MM, Bossdorf O, Allan E, Zhang Y-Y, Prati D, Blüthgen N, Boch S, Böhm S, Börschig C, *et al.* 2015. Grassland management intensification weakens the associations among the diversities of multiple plant and animal taxa. *Ecology* 96: 1492–1501.

McNaughton SJ. 1984. Grazing lawns: animals in herds, plant form, and coevolution. *The American Naturalist* 124: 863–886.

Milla R, Reich PB. 2007. The scaling of leaf area and mass: the cost of light interception increases with leaf size. *Proceedings of the Royal Society B — Biological Sciences* 274: 2109–2114.

Newbold T, Hudson LN, Hill SLL, Contu S, Lysenko I, Senior RA, Börger L, Bennett DJ, Choimes A, Collen B, *et al.* 2015. Global effects of land use on local terrestrial biodiversity. *Nature* 520: 45–50.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, *et al.* 2017. vegan: Community Ecology Package.

Petit C, Freville H, Mignot A, Colas B, Riba M, Imbert E, Hurtrez-Bousses S, Virevaire M, Olivieri I. 2001. Gene flow and local adaptation in two endemic plant species. *Biological Conservation* 100: 21–34.

Poorter H, Niinemets Ü, Poorter L, Wright IJ, Villar R. 2009. Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *The New Phytologist* 182: 565–588.

Primack RB, Antonovics J. 1981. Experimental ecological genetics in *Plantago*. V. Components of seed yield in the ribwort plantain *Plantago lanceolata* L. *Evolution* 35: 1069–1079.

R Development Core Team. 2008. R: A Language and Environment for Statistical Computing.

Reich PB, Ellsworth DS, Walters MB, Vose JM. 1999. Generality of leaf trait relationships: a test across six biomes. *Ecology* 80: 1955–1969.

Sagar GR, Harper JL. 1964. *Plantago major* L. *P. media* L. and *P. lanceolata* L. *The Journal of Ecology* 52: 189–221.

Sala OE, Chapin FS 3rd, Armesto JJ, Berlow E, Bloomfield J, Dirzo R, Huber-Sanwald E, Huenneke LF, Jackson RB, Kinzig A, *et al.* 2000. Global biodiversity scenarios for the year 2100. *Science* 287: 1770–1774.

Scheepens JF, Frei ES, Stöcklin J. 2010. Genotypic and environmental variation in specific leaf area in a widespread Alpine plant after transplantation to different altitudes. *Oecologia* 164: 141–150.

Shipley B, Almeida-Cortez J. 2003. Interspecific consistency and intraspecific variability of specific leaf area with respect to irradiance and nutrient availability. *Écoscience* 10: 74–79.

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

Soliveres S, van der Plas F, Manning P, Prati D, Gossner MM, Renner SC, Alt F, Arndt H, Baumgartner V, Binkenstein J, *et al.* 2016. Biodiversity at multiple trophic levels is needed for ecosystem multifunctionality. *Nature* 536: 456–459.

Stapledon RG. 1928. Cocksfoot Grass (*Dactylis glomerata* L.): Ecotypes in relation to the biotic factor. *The Journal of Ecology* 16: 71–104.

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.

van Tienderen PH, van der Toorn J. 1991. Genetic differentiation between populations of *Plantago lanceolata*. II. Phenotypic selection in a transplant experiment in three contrasting habitats. *The Journal of Ecology* 79: 43–59.

van Tienderen PH, van der Toorn J. 1991. Genetic differentiation between populations of *Plantago lanceolata*. I. Local adaptation in three contrasting habitats. *The Journal of Ecology* 79: 27–42.

Vellend M, Geber MA. 2005. Connections between species diversity and genetic diversity. *Ecology Letters* 8: 767–781.

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. *The Journal of Ecology* 105: 1290–1297.

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.

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

Wolff K, Van Delden W. 1989. Genetic analysis of ecological relevant morphological variability in *Plantago lanceolata* L. IV Response and correlated response to bidirectional selection for leaf angle. *Heredity* 62: 153–160.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornelissen JHC, Diemer M, *et al.* 2004. The worldwide leaf economics spectrum. *Nature* 428: 821–827.

Table 1. Results of mixed-effect models testing for heritable variation among and within 61 natural populations of *Plantago lanceolata*, for phenotypic traits measured in a common environment. The population effects are estimated using family as a random factor, and the family effects are based on four seed families per population. *P*-values are in bold if $P<0.05$ after FDR-correction. The loadings of the first two axes of a principal component analysis are also shown.

	Population		Seed family	Variance Components		PCA loadings	
	F-ratio	*P*-value	*P*-value	Among populations	Among families	PC1 (34.9%)	PC2 (20.4%)
Leaf shape	1.164	0.221	**0.008**	0.02	0.11	-0.38	-0.11
Leaf length	1.662	**0.005**	**0.003**	0.07	0.12	-0.54	0.05
Aboveground biomass	2.082	**0.000**	**0.001**	0.11	0.13	-0.24	0.12
# Inflorescences	1.671	**0.005**	**0.008**	0.06	0.10	0.17	-0.43
Onset of flowering	1.658	**0.006**	**0.000**	0.09	0.26	0.06	0.56
Reproductive allocation	1.154	0.236	**0.001**	0.02	0.14	-0.03	-0.55
Specific leaf area	2.152	**0.000**	**0.003**	0.11	0.12	0.29	-0.35
Plant height	1.545	**0.016**	**0.000**	0.06	0.19	-0.48	-0.24
Growth habit	1.336	0.076	**0.000**	0.04	0.14	-0.39	-0.05

Table 2. Summaries of linear models testing the effects of mowing, fertilisation or grazing intensities on the population means of six phenotypic traits harbouring heritable variation in *Plantago lanceolata*. *P*-values are in bold if $P<0.05$ after FDR-correction. To show the contrasting effects of mowing/fertilisation vs. grazing, positive regression slopes are highlighted in blue, and negative ones in red.

	Mowing			Fertilisation			Grazing		
	R^2	Est.	*P*-value	R^2	Est.	*P*-value	R^2	Est.	*P*-value
Plant height	0.109	0.151	**0.010**	0.000	0.006	0.885	0.036	-0.072	0.145
Leaf length	0.140	0.157	**0.003**	0.026	0.044	0.217	0.044	-0.073	0.106
Aboveground biomass	0.186	0.185	**0.001**	0.100	0.088	0.013	0.038	-0.069	0.134
# Inflorescences	0.004	-0.029	0.625	0.005	0.023	0.605	0.008	0.031	0.509
Onset of flowering	0.038	-0.083	0.131	0.004	-0.018	0.625	0.03	0.060	0.184
Specific leaf area	0.219	-0.205	**0.000**	0.060	-0.070	0.057	0.081	0.103	0.026

Table 3. Summaries of linear models testing the effects of mowing, fertilisation or grazing intensities on population-level phenotypic diversities of nine traits in *Plantago lanceolata*. None of the results were significant after FDR-correction of the *P*-values. To show the contrasting effects of mowing/fertilisation vs. grazing, positive regression slopes are highlighted in blue, and negative ones in red.

	Mowing			Fertilisation			Grazing		
	R^2	Est.	*P*-value	R^2	Est.	*P*-value	R^2	Est.	*P*-value
Leaf shape	0.016	0.036	0.337	0.008	0.017	0.498	0.001	-0.007	0.834
Leaf length	0.002	0.014	0.713	0.002	0.008	0.733	0.039	-0.048	0.125
Aboveground biomass	0.036	-0.052	0.145	0.074	-0.049	0.034	0.003	-0.012	0.685
# Inflorescences	0.100	-0.099	0.013	0.058	-0.049	0.061	0.045	0.055	0.102
Onset of flowering	0.072	-0.088	0.037	0.032	-0.038	0.166	0.089	0.081	0.02
Reproductive allocation	0.079	-0.091	0.028	0.027	-0.035	0.202	0.061	0.066	0.054
Specific leaf area	0.149	-0.101	0.002	0.071	-0.046	0.038	0.064	0.055	0.049
Plant height	0.015	-0.048	0.348	0.005	-0.017	0.606	0.015	0.041	0.341
Growth habit	0.003	-0.014	0.701	0.013	0.02	0.379	0.002	0.009	0.748

Figure 1. PCA biplot based on maternal seed family-level means of nine phenotypic traits measured in *Plantago lanceolata* in a common environment. Three land-use component vectors are fitted to show correlations between these and the phenotypes.

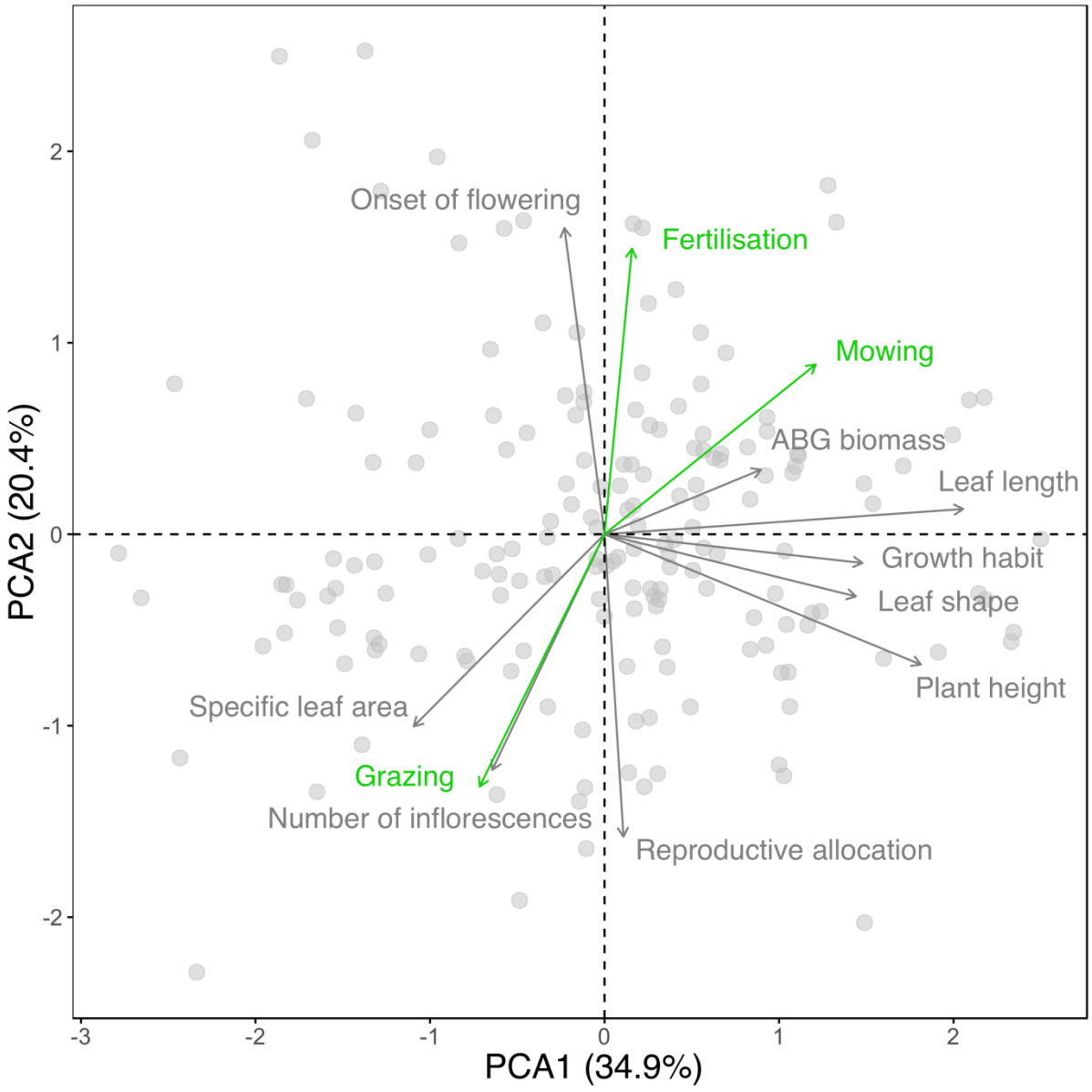


Figure 2. Relationships between land use intensities and average specific leaf area across 60 grassland populations of *Plantago lanceolata*. Mowing and grazing intensities are calculated from the yearly frequency of mowing and grazing animals per hectare, respectively. The fitted generalised linear models (GLMs) and 95% confidence intervals are shown as solid lines and grey shading, respectively.

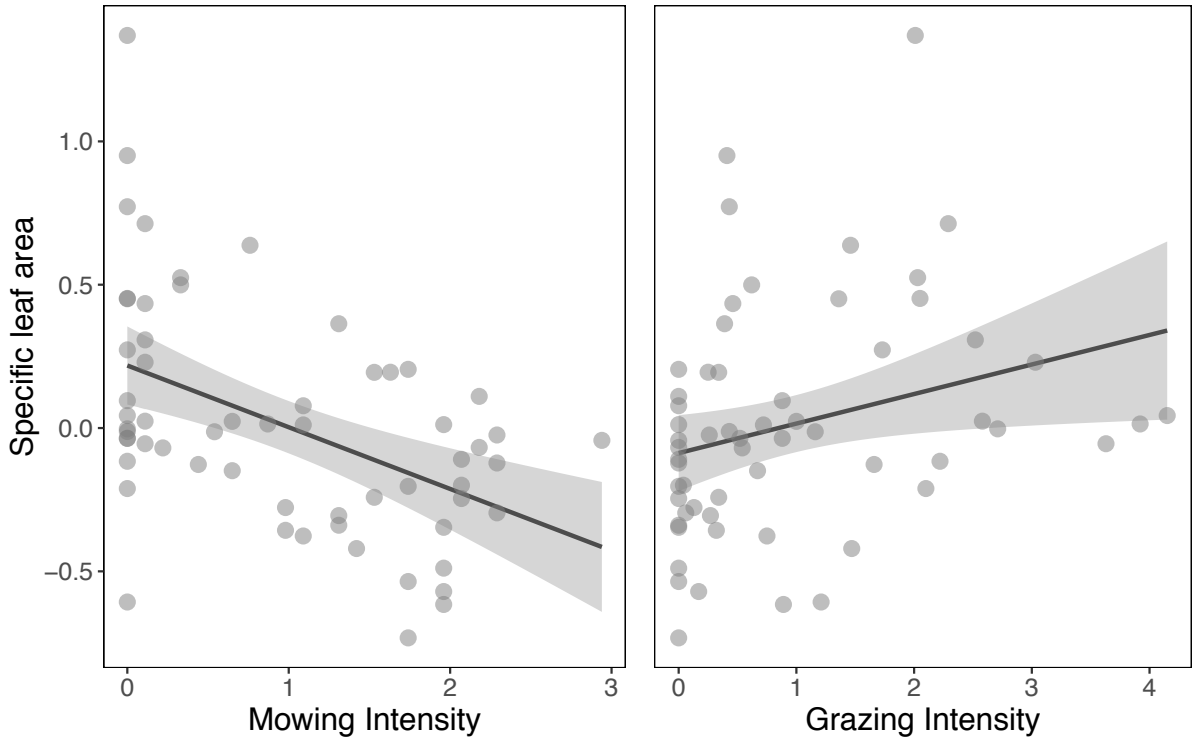
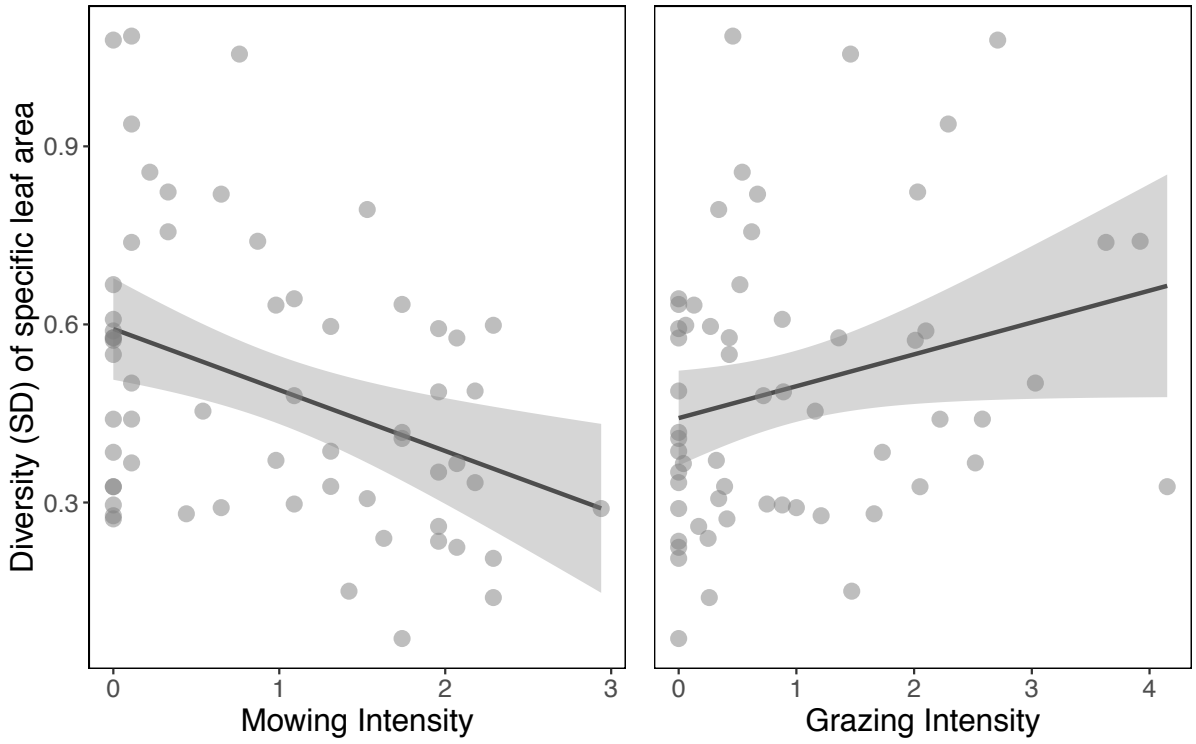


Figure 3. Relationships between land use intensities and the within-population diversities of specific leaf area across 60 grassland populations of *Plantago lanceolata*. Mowing and grazing intensities are calculated from the yearly frequency of mowing and grazing animals per hectare, respectively. The fitted generalised linear models (GLMs) and 95% confidence intervals are shown as solid lines and grey shading, respectively.



SI Table S1. Standardised land-use information of the study sites calculated from the frequency of mowing (y^{-1}), amount of nitrogen added to the plots (kg ha^{-1} y^{-1}), and density of grazing animals (livestock unit ha^{-1} y^{-1}), averaged across the years 2006–2014.

Schwäbische Alb				Hainich-Dün				Schorfheide-Chorin			
Plot ID	Mowing	Fertilisation	Grazing	Plot ID	Mowing	Fertilisation	Grazing	Plot ID	Mowing	Fertilisation	Grazing
AEG02	2.85	7.56	0	HEG05	1.97	3.51	0.95	SEG06	0.39	0	1.67
AEG03	1.97	1.17	0.05	HEG06	1.47	1.75	0.40	SEG13	1.96	0.71	0.17
AEG12	2.16	2.43	0	HEG07	0.20	0.03	2.43	SEG25	1.97	0	0
AEG13	2.06	2.54	0	HEG09	0	0	0.55	SEG30	1.77	0	0
AEG14	1.97	3.71	0	HEG11	0.98	0.96	0.13	SEG31	1.77	0	0
AEG15	2.95	5.36	0	HEG14	1.28	3.40	0.40	SEG32	1.77	0	0
AEG17	2.26	1.70	0	HEG17	0.10	0	0.41	SEG33	0.33	1.08	2.03
AEG23	1.77	0.34	0	HEG18	0.11	0	0.46	SEG34	0.49	1.67	1.15
AEG25	0	0.17	0.41	HEG20	0	0	0.87	SEG36	0	0	2.17
AEG26	0	0	2.01	HEG26	1.18	0.63	0	SEG37	0.2	0	2.84
AEG27	0	0	1.20	HEG27	1.28	1.91	0.04	SEG38	0.88	0	3.98
AEG29	1.08	0.45	0.73	HEG29	1.57	1.16	0.25	SEG39	0.59	0.2	0.76
AEG30	0.79	0.31	1.41	HEG34	1.47	2.01	0.40	SEG40	0	0	4.28
AEG31	0.69	0	0.99	HEG41	0	0	0.87	SEG41	0.10	0	4.20
AEG33	0	0	1.27	HEG43	0.39	0	0.61	SEG44	0	0.32	2.09
AEG35	2.16	1.34	0	HEG44	0.29	0	0.54	SEG45	0	0	1.81
AEG38	1.97	0.24	0	HEG45	0	0	0.47	SEG47	0.10	0	2.30
AEG39	2.06	1.90	0	HEG48	1.18	0.29	0.69	SEG48	0.10	0	2.81
AEG40	2.36	1.63	0.23	HEG49	1.38	1.22	0.24	SEG49	0	0	2.61
AEG41	2.36	4.62	0.05	HEG50	0.98	1.36	0.32	SEG50	0	0	1.98
AEG42	1.47	2.10	1.34								

SI Table S2. Sample sizes in the final analyses (initial N = 741) and summary statistics of the raw data

	N			Trait summary statistics				Variance components		
	Individuals	Families	Populations	Mean	SD	Min	Max	Pop	Fam	Residual
Leaf shape	731	247	61	9.2	2.18	4.79	16.9	0.02	0.11	0.87
Leaf length	730	247	61	26.3	4.13	13	44	0.07	0.12	0.82
Aboveground biomass	731	247	61	8.44	2.25	0.35	14.22	0.11	0.13	0.77
# Inflorescences	731	247	61	13.39	5.58	0	36	0.06	0.1	0.83
Onset of flowering	654	231	61	58.87	8.29	45	85	0.09	0.26	0.65
Reproductive allocation	731	247	61	0.42	0.17	0	0.71	0.02	0.14	0.84
Specific leaf area	731	247	61	39.44	10.83	19.07	83.82	0.11	0.12	0.78
Plant height	701	241	61	53.62	8.68	11	75	0.06	0.19	0.75
Growth habit	731	247	61	0.48	0.16	0.11	1.11	0.04	0.14	0.82

Chapter IV

Intraspecific variation in land use-related functional traits in *Plantago lanceolata*

Bence Gáspár, Oliver Bossdorf, Madalin Parepa

Abstract

Intraspecific variation in functional traits is essential for the evolutionary success of organisms. The co-variation between trait variation and environment, as well as between different traits, can help us to understand which ecological factors drive habitat adaptation, and to what extent adaptation may be constrained by trait correlations and trade-offs. In managed grasslands, plants experience a combination of competition, recurrent biomass damage and nutrient pulses. Each of these ecological challenges requires specific plant tolerances, and populations should locally adapt if intraspecific variation exists in these traits. Here, we studied variation in land use-related traits in the common grassland plant *Plantago lanceolata*. In a common environment, we quantified the competitive ability (*R**), clipping tolerance and responses to a nitrogen pulse of plants from 54 populations with different land use intensities across Germany. We found significant population differentiation in competitive ability but there was little evidence that trait variation was related to land use intensity. There was a positive relationship between competitive ability and clipping tolerance at the population level, indicating a genetic, and possibly functional, link between these two traits. In contrast, clipping tolerance and nitrogen responses were negatively correlated at the levels of plant individuals, indicating a physiological trade-off between plant responses to these two land-use processes. Our results show that there is substantial intraspecific variation in some of the key functional traits for plant success in managed grasslands, and that rapid evolution and adaptation is therefore possible in these traits.

Key words: competitive ability, fertilisation, genetic differentiation, grassland management, grazing tolerance, mowing, nutrient pulses, rapid evolution.

Introduction

Understanding evolution in response to land use in grassland plants is of great interest because of the wide distribution and economic importance of these ecosystems, and because land use change is the strongest driver of global change (Foley *et al.*, 2005, Díaz *et al.*, 2019). Already from the early 20th century, grassland researchers showed that different management regimes resulted in rapid evolutionary changes in a range of grassland species. For instance, in a common-garden collection of over 400 *Dactylis glomerata* ecotypes, Stapledon (1928) found that there were persistent growth form differences between plants from different kinds of pastures and meadows. Later, Warwick and Briggs, in their classic studies on the "genecology of lawn weeds", found similar results for several grassland species, e.g. dwarf, prostrate morphotypes originating from frequently mown lawns, and more erect ones in neighbouring populations that lacked the frequent mowing (Warwick & Briggs 1978, 1979). Evolution in response to land use was also found in the famous long-term Park Grass Experiment where Snaydon and Davies (1976) demonstrated local adaptation of *Antoxanthum odoratum* to different fertilisation and liming treatments (see also Davies & Snaydon 1973, 1976). In all of these classic studies, however, researchers compared simple categories of land use such as pastures versus meadows, or different types of fertilisation regimes, whereas finer-resolution analyses of land use processes are still rare. Moreover, previous studies usually focused on traits relevant for agriculture, such as yield, growth form and phenology, whereas other ecologically relevant functional traits received less attention.

From a plant eye's view, three of the key processes in grasslands are (1) competition with neighbouring plants, (2) the temporary nutrient pulses created by animal droppings or fertilisation, and (3) the regular disturbance and biomass removal imposed by mowing or grazing. The abilities of plants to compete with neighbours, exploit nutrient pulses, and tolerate biomass removal are thus important functional traits in grassland plants. First, competition ultimately reduces the survival, growth or reproduction of an individual plant (Aarsen & Keogh, 2002). Interspecific differences in competitive ability have been widely documented in the literature (see

for example competitive hierarchies, Keddy, 1990; variation in plant communities, Aarssen, 1992; or species coexistence, Tokeshi, 2009). Plant competitive ability can be measured in many ways (Aarssen & Keogh, 2002), and at the species level, it appears to be particularly their ability to persist at low nutrient levels that makes plants outcompete others (resource ratio hypothesis; Tilman 1985). The significance of the so-called *R** value of species – the lowest resource level that allows persistence – has been proven by many experimental studies at the species level (Wilson *et al.*, 2007). Competitive ability measured by other means has been examined at the intraspecific level, and although the existence of a genetic component and selective agency of neighbouring plants has been established, it is hard to assess the evolutionary implications of competition because of the many interacting – abiotic and biotic – factors (Cheplick, 2015); *R**, however, has not been examined at the intraspecific level so far. Second, many ecosystems experience fluctuating resource availability e.g. in the shape of snowmelt, seasonal weather events, fires, mass fruiting of plants (Ostfeld & Keesing, 2000), and human activity is especially associated with such pulses, either indirectly through the exacerbation of extreme climatic events (Coumou & Rahmstorf, 2012), or more directly through intentional nutrient deposition in agricultural landscapes. These pulsed resources have profound effects on population dynamics across entire communities and trophic networks (Gratton & Denno, 2003; Yang *et al.*, 2008) as well as across generations (Miao *et al.*, 1991). Plants often benefit from them (Bilbrough & Caldwell, 1997), and they can even lead to the promotion of invasive species (Parepa *et al.*, 2013). Plant responses to nutrient pulses have nevertheless not been investigated so far on the intraspecific level. Third, while the radical removal of plant biomass clearly reduces fitness, moderate herbivory can leave it unaffected, or in the case of overcompensation, it can even lead to increased fitness (McNaughton, 1983; Strauss & Agrawal, 1999). Tolerance to biomass damage has been extensively researched, and heritable variation within and among natural populations have been repeatedly demonstrated (e.g. Bergelson & Crawley, 1992; Agrawal, 1998; Strauss & Agrawal, 1999; Johnson, 2011), although rarely in relation to land use (but see Lennartsson *et al.*, 1997, 1998).

While all of the three functional traits are expected to be important for success in managed grasslands, it seems unlikely that plants can evolve towards improvement in all of them simultaneously. Increased competitive ability (= lower *R**) requires greater resource-efficiency, while stronger responses to nutrient pulses are only possible if plants are on the faster (= less resource-efficient) side of the fast-slow plant economy spectrum (Reich, 2014). Tolerance to biomass removal is usually based on belowground storage of resources, which also means that part of the resources is not available for other purposes anymore. In general, we should expect evolutionary trade-offs (Agrawal *et al.*, 2010) between the three functional traits, and that the specific phenotypes evolving in different grasslands depend on the intensities of fertilisation versus mowing and grazing damage.

We addressed these questions the framework of the Biodiversity Exploratories project (www.biodiversity-exploratories.de), a large-scale and long-term network of ecological study sites for understanding the relationships between land use, biodiversity and ecosystem functioning. The project includes 150 grassland plots across Germany (Fischer *et al.*, 2010), with 50 plots in each of the three regions Schorfheide-Chorin (northern Germany), Hainich-Dün (central Germany) and Schwäbische Alb (southwest Germany). Within each region, the plots cover a broad range of land use types and intensities. The level of detail of the land use information available for these plots, with precise data on mowing frequencies, livestock densities and amounts of fertilisation, obtained from annual surveys (Blüthgen *et al.*, 2012), is a unique feature of the Biodiversity Exploratories project and, together with the large number of plots, makes it a powerful study system for testing rapid evolution in managed grasslands.

There is already some previous evidence from the Biodiversity Exploratories for phenotypic evolution in response to land use in several grassland plant species (Kloss *et al.*, 2011; Völler *et al.*, 2013, 2017). Here, we built on these studies and examined 54 populations of the common grassland species *Plantago lanceolata*. Unlike the previous studies, which only conducted simple phenotyping in a common environment, we carried out a greenhouse experiment with a complex series of treatments (Fig. 1) which allowed us to quantify the *R** values of plants, as well as

their pulse response and clipping tolerances. Specifically, we asked the following questions: (1) Is there intraspecific variation in the aforementioned three functional traits in *P. lanceolata*? (2) What is the relationship between land use and the variation in these traits? (3) Are there trade-offs between the three traits, and are these trade-offs influenced by land use intensity?

Materials and Methods

Study species and experimental design

To test the questions outlined above, we worked with *Plantago lanceolata* L. (Plantaginaceae), a short-lived perennial rosette herb that is very common in European grasslands and grows under a wide range of environmental conditions. *P. lanceolata* is also one of the most common plant species in the Biodiversity Exploratories, occurring on over 100 of the 150 grassland plots. In September 2015, we collected ripe seeds of *P. lanceolata* in each of the three regions, and from the broadest possible land-use gradient in each (Supplementary Table S2). Altogether, we sampled seeds from 54 plots, with 5–12 individual plants per plot.

We stratified the seeds at 5°C under moist and dark conditions for three weeks (Pons, 1992) and transplanted the germinated seedlings to 1-L pots filled with a 7:1.5:1 mixture of nutrient-poor soil, vermiculite and sand, with 5–12 individuals per population and a total of 540 plants (Supplementary Table S2). The pots were placed in a climate-controlled greenhouse with temperature set to 21°C/15°C at a 16h/8h day/night cycle. After six weeks, we rearranged all pots into a randomised block design, and we then let the plants grow for another seven weeks to ensure strong nutrient depletion in all pots (Fig. 1). At this point, we took a 5 cm^3 soil sample from each pot that was later analysed for total nitrogen content with a EuroEA Elemental Analyser (HEKAtech, Wegberg , Germany) at the Soil Biogeochemistry Lab at Karlsruhe Institute of Technology, and we measured the chlorophyll content of two leaves on each plant with a SPAD 502 chlorophyll meter (Konica-Minolta, Tokyo, Japan). After that, we fertilised each plant with 10 ml of liquid NPK fertiliser (Wuxal Universaldünger; Hauert MANNA Düngerwerke GmbH, Nürnberg, Germany) at a concentration equivalent to 50 kg N/ha. Ten days later, we measured chlorophyll

content again on two newly grown leaves of each plant. Two weeks after adding the fertiliser, we clipped all plants one centimetre above ground. After another three weeks, we harvested the aboveground biomass of all plants, dried it at 70°C for three days, and weighed it.

Data Analysis

Our data analyses generally focused on three variables: (1) the competitive ability of each plant, estimated as *1-R** (Tilman, 1985) where *R** was the fraction of total nitrogen in the potting soil left after 11 weeks of growth, (2) the pulse response as the ratio between the leaf chlorophyll contents after and before the fertilisation, with higher values indicating more successful utilisation of the added nitrogen, and (3) the clipping tolerance of plants, calculated as the ratio between their aboveground biomass from the second and first harvest, again with higher values indicating faster recovery from clipping damage.

Prior to the main analyses, we simplified our data by removing some non-relevant sources of variation from it. We fitted linear models with the three regions of the Biodiversity Exploratories and the blocks in the greenhouse as fixed factors to each dependent variable, and we used the residuals from these models for all subsequent analyses (Manning *et al.*, 2015; Soliveres *et al.*, 2016). To improve the normality of error distributions, the data for pulse response and clipping tolerance were additionally log-transformed.

First, we tested for intraspecific variation in the three focus traits with mixed-effect models that included populations as fixed factors and maternal seed families nested within populations as random factors (Zuur *et al.*, 2009; see Supplementary Information Table S1 for model formulas). Second, we tested for relationships between land use and the three traits by fitting separate mixed models for each combination of land use intensities (mowing, fertilisation, grazing) and trait (competitive ability, pulse response, clipping tolerance), with each model including one of the land use intensities as explanatory variable plus population and maternal seed families nested within populations as random factors (Table S1). Next, we tested for trade-offs between the three focus traits by examining their statistical

relationships at the level of individuals, seed families and populations. At the individual-level, we fitted mixed models with random intercept and slope that included the respective other trait as explanatory variable, plus population and family nested within population as random factors. At the family level, we analysed family means and included only population as random factor, and at the population level, we used simple linear models regressing the population means of two traits against each other. In the cases where we found significant relationships between the traits, we proceeded to the final step in our analyses. IN this step, we tested whether land use affected trait relationships through a series of mixed models with random intercept and slope that included the respective other trait, one of the three land use intensities, and their interactions, as fixed factors, plus populations and families nested within populations as random factors. All statistical analyses were done in R (R Development Core Team, 2008). We corrected all *P*-values for false discovery rates.

Results

We found significant heritable variation, both at the population and seed family level, for competitive ability, but only marginally significant family-level variation in clipping tolerance, and no significant variation at all in pulse response (Table 1 and Figure 2). There were no significant relationships at all between land use intensity and the three studied functional traits (Table 2). When we tested for relationships between competitive ability, pulse response and clipping tolerance, we found significant negative relationships between pulse response and clipping tolerance at the level of individuals and seed families, and a significant positive relationship between competitive ability and clipping tolerance at the population level (Table 3 and Fig 3). Furthermore, we found a significant effect of mowing on the individual-level relationship between pulse response and clipping tolerance ($F = 9.08$, $P = 0.025$ for mowing x pulse response interaction), with the negative relationship between the two traits disappearing at higher mowing intensities (Fig 4). There were no other significant land use effects on trait relationships.

Discussion

To understand plant intraspecific variation in relation to land use, we studied 54 grassland populations of *Plantago lanceolata* that strongly differed in their intensities of mowing, grazing and fertilisation. We specifically examined three functional traits that we expected to be important for plant survival in such grasslands: competitive ability, clipping tolerance and the ability of plants to quickly respond to nutrient pulses. We found substantial intraspecific variation in competitive ability (*R**) but not in the other two traits, and there was no evidence for population-level relationships between traits and land-use intensity. However, there were several positive or negative relationships between functional traits at the levels of individuals, families or populations, indicating physiological or evolutionary links between these traits. Below, we discuss the results in detail, and attempt to place them into a broader context.

Intraspecific variation

A necessary prerequisite for genetic differentiation and local adaptation in the examined traits is that significant intraspecific variation exists in this system. We did not find any significant family- or population-level variation in clipping tolerance and plant responses to a nutrient pulse, but there was substantial intraspecific variation in *R** competitive ability, both at the level of seed families and populations. To our knowledge, this is the first time that intraspecific variation, and thus microevolution, in this type of competitive ability has been studied and demonstrated in plants.

We were somewhat surprised to not find population differentiation in clipping tolerance because intraspecific variation has been previously shown in other plant species (e.g. Agrawal *et al.*, 1999; Johnson, 2011; Juenger & Bergelson, 2000; Strauss & Agrawal, 1999; Deng *et al.* unpublished). We also did not find population differentiation in pulse response, and in this trait, we also do not know about previous studies on intraspecific variation. With 54 populations and 199 seed families, a lack of statistical power is an unlikely explanation in our case. Instead, we think that it was rather a combination of weak true patterns and high signal-to-noise ratio. First, since *Plantago lanceolata* is wind-pollinated and self-incompatible (Kuiper

& Bos, 1992), there is generally strong gene flow and relatively weak population differentiation in this species (Gáspár *et al.* 2019). Second, we worked with an F_1 generation that had random fathers (from the field) but that, unlike under field conditions, was not experiencing strong natural selection. This likely further increased variation among individuals and therefore lowered the signal-to-noise ratio in our system. Finally, clipping tolerance and pulse response are both derived traits based on several, error-prone measurements, and thus error propagation could have further added to this problem. However, in spite of all this, we did find significant family- and population-level variation in *R**, which underlines the ecological and evolutionary significance of this result.

No relationships with land use

We found no relationships between the land-use intensities recorded in the Biodiversity Exploratories and the three studied functional traits. This contrasts with previous studies in the Biodiversity Exploratories (Völler *et al.*, 2013, 2017) as well as in other systems that demonstrated land use-related phenotypic changes in plants (e.g. Aarssen & Turkington, 1985a, b, c, 1987; Lennartsson *et al.*, 1997; Briggs, 2009). In principle, there are three possible explanations: (1) a true pattern could not be detected because of statistical or methodological shortcomings, (2) there was no pattern yet because the land use has not been acting long enough yet in our system, or (3) there is no pattern. As already explained above, our study did not lack statistical power, and it covered a broad range of land use intensities, also compared to previous studies. Moreover, although there is some interannual variation in land use in the Biodiversity Exploratories (Blüthgen *et al.*, 2012; Allan *et al.*, 2014), which could potentially impede the impacts of natural selection, previous studies already found land use-related differentiation of plant phenotypes in our system (Völler *et al.*, 2013, 2017). It is also known from other studies that that a couple of years can be enough for stable shifts in plant phenotypes between differential management (Briggs, 2009). Therefore, explanations (1) and (2) both appear unlikely, and we need to consider the third option that there might simply be no relationships between land use and the three studied functional traits; possibly because of error

propagation and the derived nature of the traits, or evolutionary constraints particular to these traits and land use in this system.

Correlations between three functional traits

Besides quantifying intraspecific variation in the three functional traits and their relationships with land use, we also tested for interrelationships between traits, and we did this at three levels: plant individuals, maternal seed families and populations. Each of these levels provides us with different answers: at the level of individuals, trait correlations are most likely related to functional-physiological constraints or necessities, whereas at the level of seed families they reflect underlying genetic correlations, and at the level of populations they rather indicate trait syndromes associated with habitat adaptation.

We found no relationships between competitive ability and pulse response at any of these levels. This was surprising as lower *R** values (i.e. better competitive ability) should be coupled to a resource-conservative plant economy, whereas strong responses to nutrient pulses require a large metabolic capacity. We therefore expected a trade-off between the two traits. However, our results suggest that competitive ability evolves fairly independently. The only observed trait correlation involving competitive ability was a positive population-level correlation between competitive ability and clipping tolerance, indicating both traits might be beneficial in the same environments. Resprouting in *Plantago lanceolata* is based on belowground resource storage (Latzel & Klimesová, 2009; Latzel *et al.*, 2014). Thus, in contrast to pulse response, both clipping tolerance and *R** competitive ability are related to resource conservation, and therefore both traits should be beneficial in the less nutrient-rich pastures or meadows which make up part of the grassland plots in the Biodiversity Exploratories. However, the two traits were not significantly correlated at the level of individuals or seed families, indicating that they are not genetically correlated, or directly linked through physiology. Another potential explanation for the lack of a family-level relationship could be the inflated genetic variation in the F_1 generation already explained above (see also Gáspár *et al.*, 2019). However, while F_1 plants from the same mother may have many different fathers,

these most likely come from the same population (Kuiper & Bos, 1992, p. 226), so that population-level differences may have been maintained, and could thus be detected, in our study.

Surprisingly, plant responses to nutrient pulses were negatively correlated to clipping tolerance at the levels of individuals and maternal seed families. Together with the lack of a population differentiation in these traits, this indicates physiological and/or genetic links between them. Again, resource economy appears to be the best explanation here. Clipping tolerance is generally thought to be more prevalent in species or genotypes with a more conservative metabolism and more root-, non-structural carbohydrate reserves, whereas a stronger response to a nutrient pulse should requires higher metabolic rate and less storage (Strauss & Agrawal, 1999; Reich, 2014). Thus, there should be classic resource trade-off between the two traits. This explanation is further supported by the fact that we found the negative correlation mainly in plants from plots with less than one mowing event per year, whereas the relationship tended to disappear at higher mowing frequencies. In the Biodiversity Exploratories, frequent mowing is usually associated with strong fertilisation (Blüthgen *et al.*, 2012). Thus, the resource trade-off disappears when resources become less limiting (Agrawal *et al.*, 2010).

Conclusions

Our study documents, for the first time, significant intraspecific variation in R^* competitive ability and that this trait appears to evolve independently of pulse response and clipping tolerances. We could identify an intrinsic, physiological trade-off between clipping tolerance and pulse response that disappeared in plants originating from more intensively managed plots. We have not found any relationship between the three traits and land use, which might be due to various factors of the management regimes resulting in inconsistent selective forces, the biology of *P. lanceolata*, and our experimental design.

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References

Aarssen LW. 1992. Causes and consequences of variation in competitive ability in plant communities. *Journal of Vegetation Science* 3: 165–174.

Aarssen LW. 2005. On size, fecundity, and fitness in competing plants. In: Reekie EG, Bazzaz FA, eds. *Reproductive Allocation in Plants*. Academic Press, 215–244.

Aarssen L, Keogh T. 2002. Conundrums of competitive ability in plants: what to measure? *Oikos* 96: 531–542.

Aarssen LW, Turkington R. 1985a. Within-species diversity in natural populations of *Holcus lanatus*, *Lolium perenne* and *Trifolium repens* from four different-aged pastures. *The Journal of Ecology* 73: 869–886.

Aarssen LW, Turkington R. 1985b. Biotic specialization between neighbouring genotypes in *Lolium perenne* and *Trifolium repens* from a permanent pasture. *The Journal of Ecology* 73: 605–614.

Aarssen LW, Turkington R. 1985c. Competitive relations among species from pastures of different ages. *Canadian Journal of Botany* 63: 2319–2325.

Aarssen LW, Turkington R. 1987. Responses to defoliation in *Holcus lanatus*, *Lolium perenne*, and *Trifolium repens* from three different-aged pastures. *Canadian Journal of Botany* 65: 1364–1370.

Agrawal AA. 1998. Induced responses to herbivory and increased plant performance. *Science* 279: 1201–1202.

Agrawal AA, Conner JK, Rasmann S. 2010. Tradeoffs and negative correlations in evolutionary ecology. In: M. A. Bell, W. F. Eanes, D. J. Futuyma, and J. S. Levinton, ed. *Evolution After Darwin: The First 150 Years*. 243–268.

Agrawal AA, Strauss SY, Stout MJ. 1999. Costs of induced responses and tolerance to herbivory in male and female fitness components of wild radish. *Evolution* 53: 1093–1104.

Allan E, Bossdorf O, Dormann CF, Prati D, Gossner MM, Tscharntke T, Blüthgen N, Bellach M, Birkhofer K, Boch S, *et al.* 2014. Interannual variation in land-use intensity enhances grassland multidiversity. *Proceedings of the National Academy of Sciences of the United States of America* 111: 308–313.

Bergelson J, Crawley MJ. 1992. The effects of grazers on the performance of individuals and populations of scarlet gilia, *Ipomopsis aggregata*. *Oecologia* 90: 435–444.

Bilbrough CJ, Caldwell MM. 1997. Exploitation of springtime ephemeral N pulses by six Great Basin plant species. *Ecology* 78: 231–243.

Blüthgen N, Dormann CF, Prati D, Klaus VH, Kleinebecker T, Hölzel N, Alt F, Boch S, Gockel S, Hemp A, *et al.* 2012. A quantitative index of land-use intensity in grasslands: Integrating mowing, grazing and fertilization. *Basic and Applied Ecology* 13: 207–220.

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

Coumou D, Rahmstorf S. 2012. A decade of weather extremes. *Nature Climate Change* 2: 491–496.

Davies, M.S. & Snaydon, R.W. 1973. Physiological differences among populations of *Anthoxanthum odoratum* L. on the Park Grass Experiment, Rothamsted. I. Response to calcium. *Journal of Applied Ecology* 10: 33– 45.

Davies, M.S. & Snaydon, R.W. 1976. Rapid population differentiation in a mosaic environment. III. Measures of selection pressures. *Heredity* 36: 59–66.

Díaz S, Settele J, Brondízio E, Ngo H, Guèze M, Agard J, Arneth A, Balvanera P, Brauman K, Butchart S, *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.

Fischer M, Bossdorf O, Gockel S, Hänsel F, Hemp A, Hessenmöller D, Korte G, Nieschulze J, Pfeiffer S, Prati D, *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, Barford C, Bonan G, Carpenter SR, Chapin FS, Coe MT, Daily GC, Gibbs HK, *et al.* 2005. Global consequences of land use. *Science* 309: 570–574.

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

Gratton C, Denno RF. 2003. Inter-year carryover effects of a nutrient pulse on *Spartina* plants, herbivores, and natural enemies. *Ecology* 84: 2692–2707.

Johnson MTJ. 2011. Evolutionary ecology of plant defenses against herbivores. *Functional Ecology* 25: 305–311.

Juenger T, Bergelson J. 2000. The evolution of compensation to herbivory in scarlet gilia, *Ipomopsis aggregata*: herbivore-imposed natural selection and the quantitative genetics of tolerance. *Evolution* 54: 764–777.

Keddy PA. 1990. Competitive hierarchies and centrifugal organization in plant communities. In: Grace JB, Tilman D, eds. *Perspectives on Plant Competition*. Academic Press, 265–290.

Kloss L, Fischer M, Durka W. 2011. Land-use effects on genetic structure of a common grassland herb: A matter of scale. *Basic and Applied Ecology* 12: 440–448.

Kuiper PJC, Bos M. 1992. *Plantago: A Multidisciplinary Study*. Springer.

Latzel V, Klimešová J. 2009. Fitness of resprouters versus seeders in relation to nutrient availability in two *Plantago* species. *Acta Oecologica* 35: 541–547.

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.

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.

Manning P, Gossner MM, Bossdorf O, Allan E, Zhang Y-Y, Prati D, Blüthgen N, Boch S, Böhm S, Börschig C, *et al.* 2015. Grassland management intensification weakens the associations among the diversities of multiple plant and animal taxa. *Ecology* 96: 1492–1501.

McNaughton SJ. 1983. Compensatory plant growth as a response to herbivory. *Oikos* 40: 329–336.

Miao SL, Bazzaz FA, Primack RB. 1991. Persistence of maternal nutrient effects in *Plantago major*: the third generation. *Ecology* 72: 1634–1642.

Ostfeld RS, Keesing F. 2000. Pulsed resources and community dynamics of consumers in terrestrial ecosystems. *Trends in Ecology & Evolution* 15: 232–237.

Parepa M, Fischer M, Bossdorf O. 2013. Environmental variability promotes plant invasion. *Nature Communications* 4: 1604.

Pons TL. 1992. Seed germination of Plantago major ssp. major and *Plantago lanceolata*. In: *Plantago: A Multidisciplinary Study*. Springer, 161–169.

R Development Core Team. 2008. R: A Language and Environment for Statistical Computing.

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

Snaydon RW, Davies MS. 1976. Rapid population differentiation in a mosaic environment. IV. Populations of *Anthoxanthum odoratum* at sharp boundaries. *Heredity* 37: 9-27.

Soliveres S, van der Plas F, Manning P, Prati D, Gossner MM, Renner SC, Alt F, Arndt H, Baumgartner V, Binkenstein J, *et al.* 2016. Biodiversity at multiple trophic levels is needed for ecosystem multifunctionality. *Nature* 536: 456–459.

Stapledon RG. 1928. Cocksfoot grass (*Dactylis Glomerata* L.): Ecotypes in relation to the biotic factor. *The Journal of Ecology* 16: 71–104.

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

Tilman D. 1985. The resource-ratio hypothesis of plant succession. *The American Naturalist* 125: 827–852.

Tokeshi M. 2009. *Species Coexistence: Ecological and Evolutionary Perspectives.* John Wiley & Sons.

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. *The Journal of Ecology* 105: 1290–1297.

Warwick SI, Briggs D. 1978. The Genecology of Lawn Weeds. I. Population differentiation in *Poa annua* L. in a mosaic environment of bowling green lawns and flower beds. *The New Phytologist* 81: 711–723.

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.

Weiner J. 1988. The influence of competition on plant reproduction. In: Lovett Doust J, Lovett Doust L, eds. *Plant reproductive ecology: patterns and strategies.* Oxford University Press, 228–245.

Wilson JB, Spijkerman E, Huisman J. 2007. Is there really insufficient support for Tilman's *R** concept? A comment on Miller *et al. The American Naturalist* 169: 700–706.

Yang LH, Bastow JL, Spence KO, Wright AN. 2008. What can we learn from resource pulses? *Ecology* 89: 621–634.

Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM. 2009. *Mixed effects models and extensions in ecology with R.* Springer.

Table 1. Results of mixed models testing for heritable variation in three functional traits in *Plantago lanceolata*, with populations and seed families included as fixed and random factors, respectively. LRT = likelihood-ratio test statistics. All *P*-values are FDR-corrected.

	Population		Seed family	
	F	*P*	*LRT*	*P*
Competitive ability	1.60	**0.049**	16.82	**0.000**
Pulse response	1.01	0.463	0.04	0.835
Clipping tolerance	1.30	0.169	4.05	0.066

Table 2. Results of mixed models testing for the effects of mowing, fertilisation and grazing on three functional traits in *Plantago lanceolata*, with populations and seed families included as random factors. All *P*-values are FDR-corrected.

	Mowing			Fertilisation			Grazing		
	Est.	*F*	*P*	Est.	*F*	*P*	Est.	*F*	*P*
Competitive ability	0.142	4.31	0.387	0.050	1.51	0.504	-0.071	1.68	0.504
Pulse response	0.014	0.08	0.838	0.001	0.04	0.838	0.009	0.06	0.838
Clipping tolerance	0.007	1.85	0.838	0.011	1.60	0.504	0.007	0.39	0.838

Table 3. Results of random slope and intercept mixed effects models testing the relationships between competitive ability (CA), pulse response (PR) and clipping tolerance (CT) in *Plantago lanceolata*, with populations and seed families included as random factors. All *P*-values are FDR-corrected, and $P<0.05$ are in bold.

	Individuals			Families			Populations		
	Est.	*F*	*P*	Est.	*F*	*P*	Est.	*F*	*P*
CA ~ PR	0.07	3.26	0.162	0.12	1.31	0.336	0.01	0.00	0.967
CA ~ CT	-0.01	0.01	0.967	0.11	1.33	0.336	0.44	6.21	**0.048**
PR ~ CT	-0.05	11.47	**0.009**	-0.05	8.81	**0.018**	-0.05	2.52	0.212

Figure 1. Schematic of the sequence and duration of experimental treatments used to estimate competitive ability (*R**), pulse response and clipping tolerance in *Plantago lanceolata* plants from 54 grasslands of different land-use intensities.

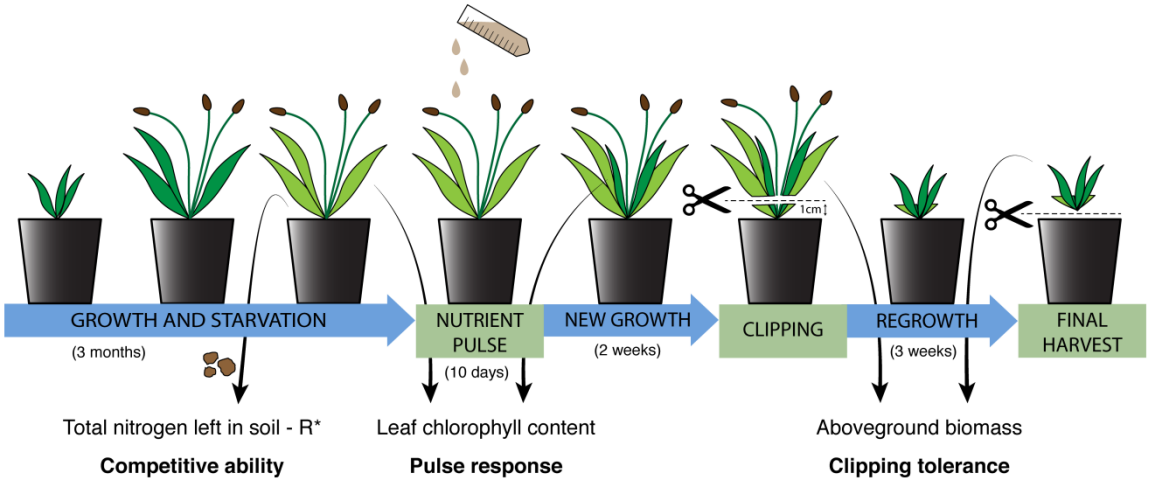


Figure 2. Variation among populations (boxplots) and maternal seed families (black dots within boxplots) in three functional traits in *Plantago lanceolata*. The boxplots are based on all individuals per population and indicate medians, 25th/75th percentiles, and the 1.5 x interquartile ranges.

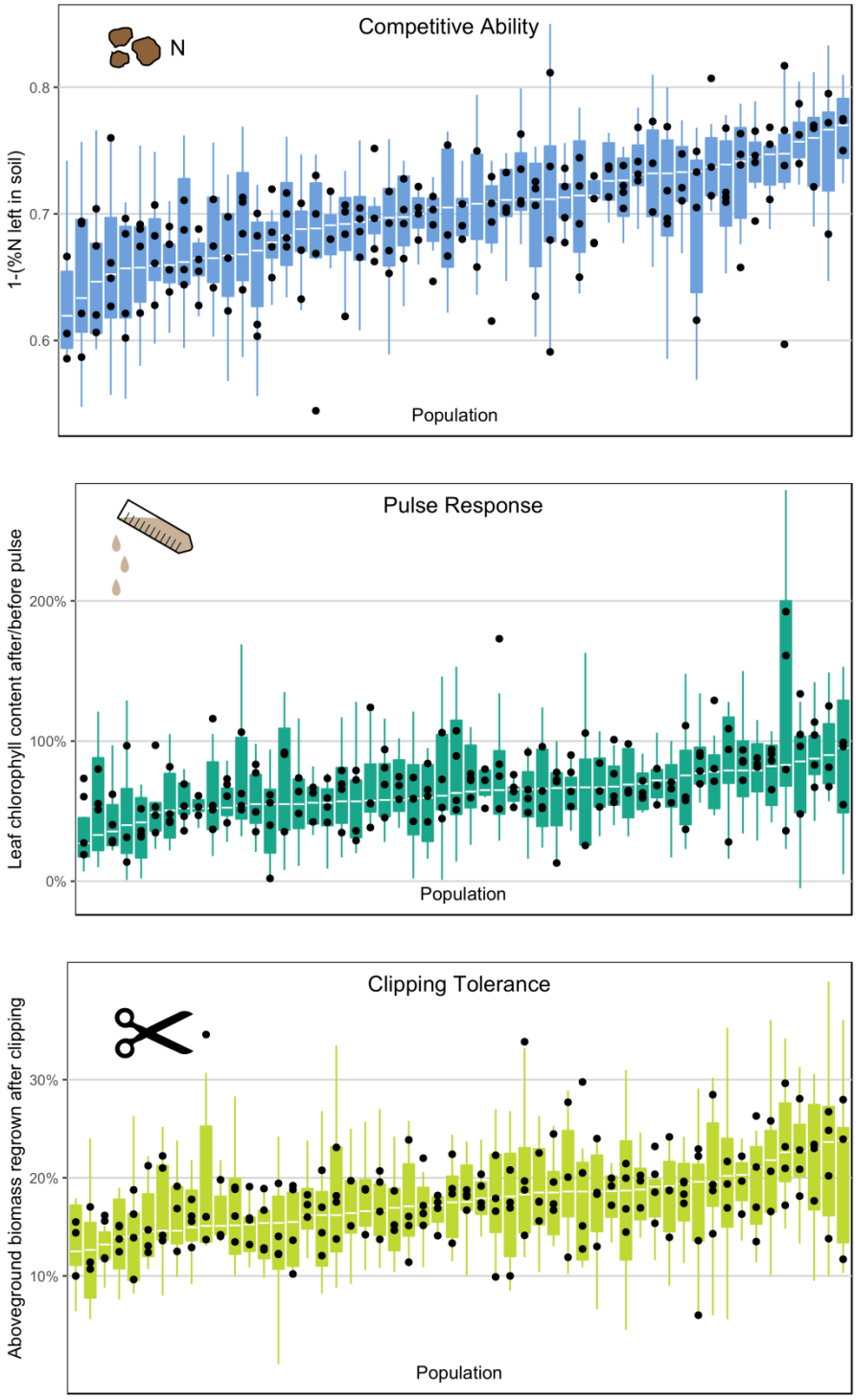


Figure 3. Relationships between the three studied functional traits of *Plantago lanceolata* at the levels of individuals, seed families and populations. Solid and dashed line plots indicate the fitted models for significant and non-significant relationships, respectively, with their 95% confidence intervals.

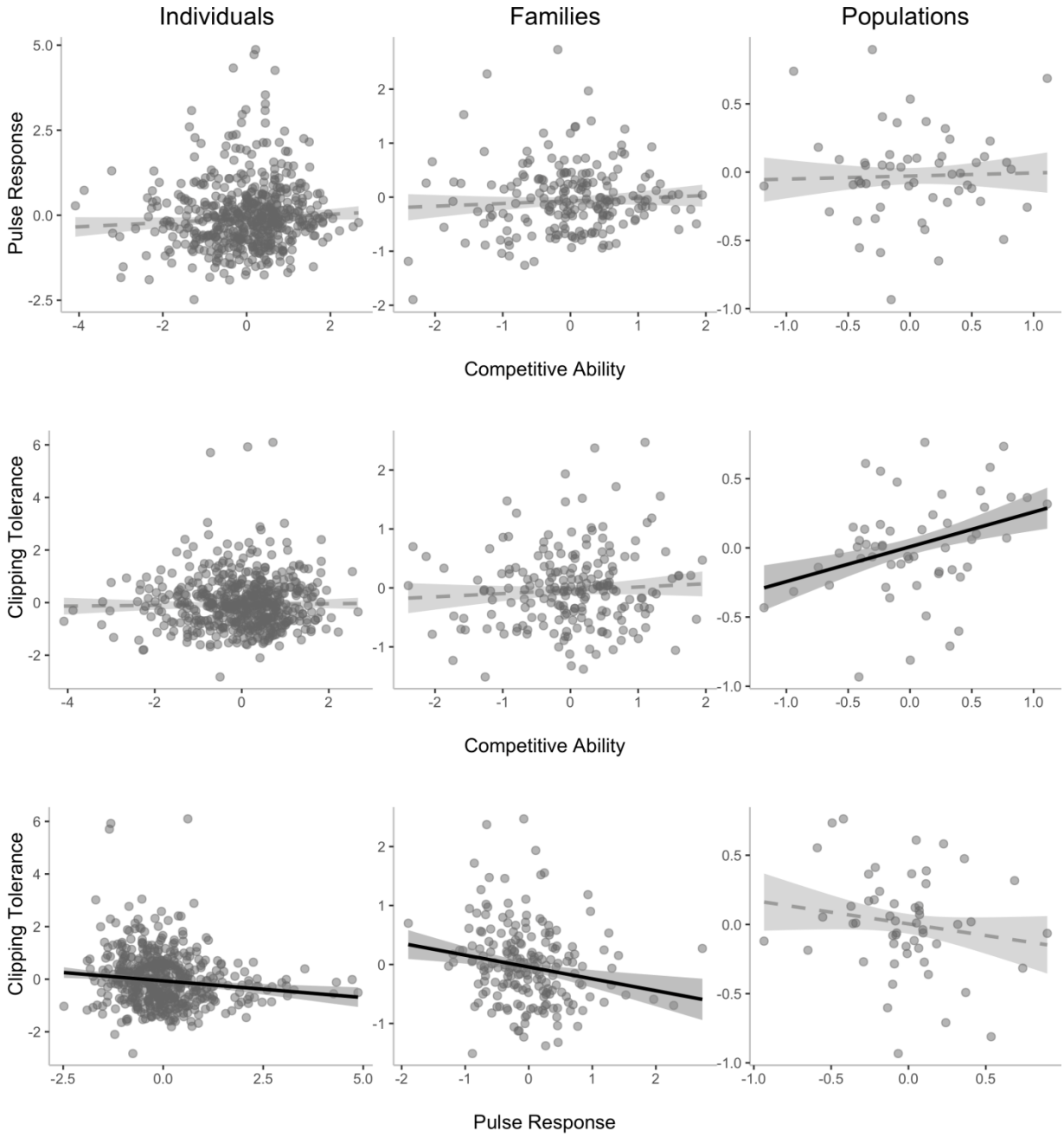
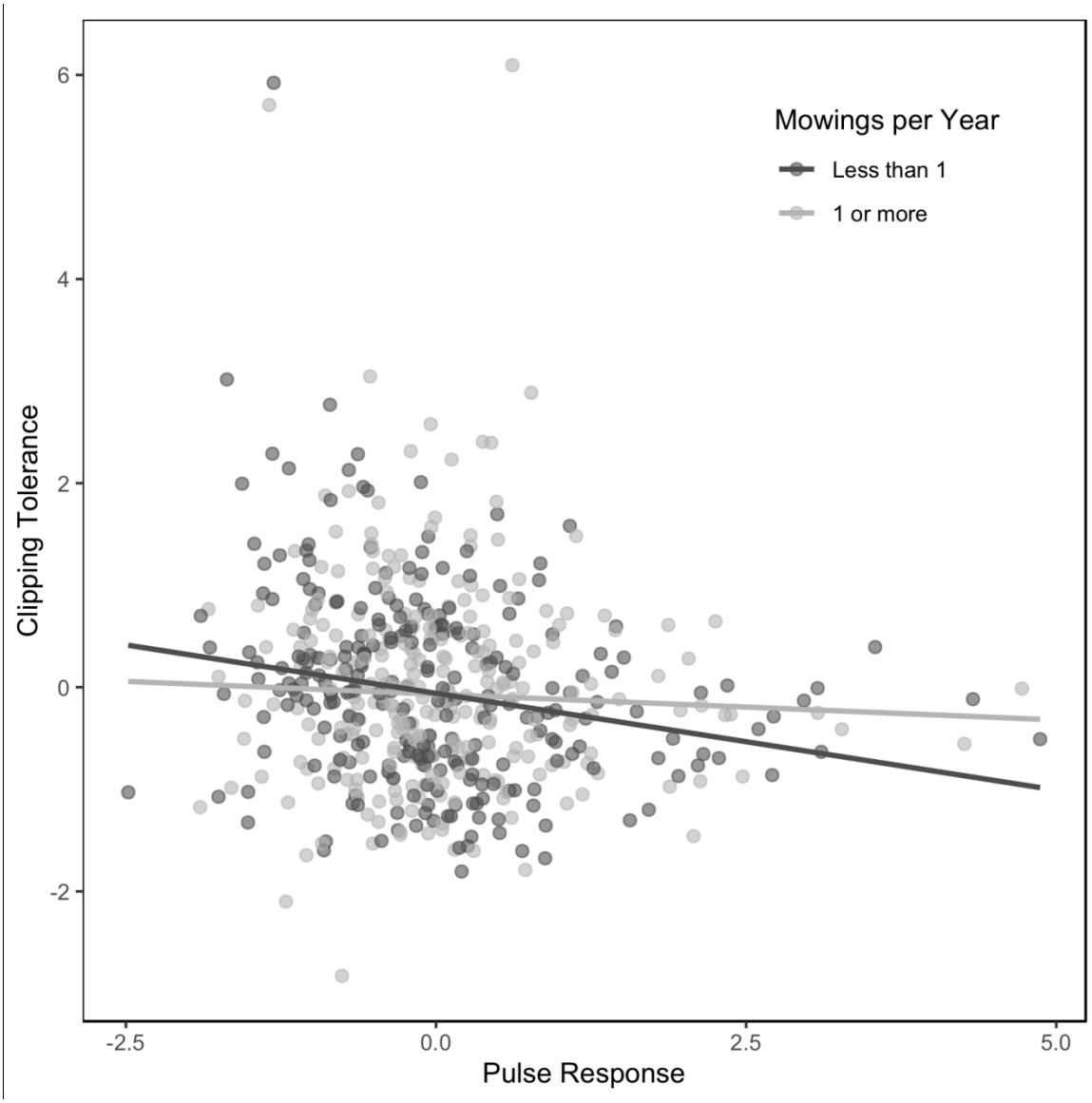


Figure 4. The mowing intensity of their grasslands of origin affects functional trait correlations in *Plantago lanceolata*. Each dot represents a plant individual grown in a common environment.



SI Table S1. Model structures used for the different data analyses. *ry* stands for the residuals obtained from model 1 for each of the three traits analysed that we used for all further analyses, indexed with *i* and *j* when two of them are included in one model.

	Model formula	Purpose
1.	lm(y ~ region + block)	Creating region- and block-effect-free data
2.	lmer(ry ~ population + (1|population:family))	Heritable intraspecific trait variation
3.	lmer(ry ~ land use + (1|population) + (1|population:family))	Land use effects on functional traits
4.	lmer(ry_i ~ ry_j + (ry_j|population) + (ry_j|population:family))	Trait correlations at individual level
5.	lmer(ry_i ~ ry_j + (ry_j|population))	Trait correlations at seed family level
6.	lm(ry_i ~ ry_j)	Trait correlations at population level
7.	lmer(ry_i ~ ry_j * land use + (ry_j|population) + (ry_j|population:family))	Effects of land use on trait correlations

SI Table S2. Land-use calculated from the frequency of mowing (y^{-1}), amount of nitrogen added to the plots (kg ha^{-1} y^{-1}), and density of grazing animals (livestock unit ha^{-1} y^{-1}), averaged across the years 2006–2014 of all 54 studied grassland populations of *Plantago lanceolata*; and the numbers of seed families, as well as individuals per family and per population, used in our greenhouse study.

Region	Plot ID	Mowing intensity	Fertilisation intensity	Grazing intensity	# Families	# Individuals				Total # Individuals
						Fam 1	Fam 2	Fam 3	Fam 4	
Schwäbische Alb	AEG02	2.85	7.56	0	3	3	2	2	NA	7
	AEG03	1.97	1.17	0.05	4	3	3	3	3	12
	AEG12	2.16	2.43	0	4	3	3	3	3	12
	AEG13	2.06	2.54	0	4	3	1	3	3	10
	AEG14	1.97	3.71	0	4	1	3	3	3	10
	AEG15	2.95	5.36	0	4	3	3	1	3	10
	AEG17	2.26	1.7	0	4	3	3	3	3	12
	AEG23	1.77	0.34	0	4	3	3	3	3	12
	AEG27	0	0	1.2	4	3	3	2	3	11
	AEG29	1.08	0.45	0.73	4	3	3	3	2	11
	AEG30	0.79	0.31	1.41	2	2	3	NA	NA	5
	AEG31	0.69	0	0.99	4	3	3	3	1	10
	AEG33	0	0	1.27	4	3	3	3	3	12
	AEG35	2.16	1.34	0	4	2	2	3	1	8
	AEG38	1.97	0.24	0	3	2	3	3	NA	8
	AEG39	2.06	1.9	0	3	3	3	1	NA	7
	AEG40	2.36	1.63	0.23	4	3	1	1	1	6
	AEG41	2.36	4.62	0.05	4	3	3	3	3	12
	AEG42	1.47	2.1	1.34	3	3	3	3	NA	9
Hainich-Dün	HEG05	1.97	3.51	0.95	4	3	3	3	2	11
	HEG06	1.47	1.75	0.4	4	2	3	3	3	11
	HEG07	0.2	0.03	2.43	4	3	3	3	3	12
	HEG09	0	0	0.55	4	2	2	3	1	8
	HEG14	1.28	3.4	0.4	3	3	3	3	NA	9
	HEG17	0.1	0	0.41	4	3	3	3	1	10
	HEG20	0	0	0.87	3	1	5	3	NA	9
	HEG26	1.18	0.63	0	4	1	3	3	3	10
	HEG27	1.28	1.91	0.04	4	3	3	3	3	12
	HEG29	1.57	1.16	0.25	3	3	2	2	NA	7
	HEG34	1.47	2.01	0.4	4	3	2	3	3	11
	HEG41	0	0	0.87	3	3	2	2	NA	7
	HEG43	0.39	0	0.61	4	3	3	1	3	10

	HEG44	0.29	0	0.54	4	3	1	1	2	7
	HEG45	0	0	0.47	3	3	3	3	NA	9
	HEG48	1.18	0.29	0.69	4	3	3	3	3	12
	HEG49	1.38	1.22	0.24	4	1	3	3	3	10
Schorfheide-Chorin	SEG06	0.39	0	1.67	4	3	3	3	1	10
	SEG25	1.97	0	0	3	3	3	3	NA	9
	SEG30	1.77	0	0	3	3	3	3	NA	9
	SEG31	1.77	0	0	4	3	3	3	3	12
	SEG32	1.77	0	0	3	3	3	3	NA	9
	SEG34	0.49	1.67	1.15	4	3	3	1	3	10
	SEG36	0	0	2.17	4	3	3	3	3	12
	SEG37	0.2	0	2.84	4	3	3	3	3	12
	SEG38	0.88	0	3.98	4	3	3	3	1	10
	SEG39	0.59	0.2	0.76	4	3	3	3	3	12
	SEG40	0	0	4.28	4	3	3	3	1	10
	SEG41	0.1	0	4.2	3	3	3	3	NA	9
	SEG44	0	0.32	2.09	4	3	3	3	2	11
	SEG45	0	0	1.81	3	3	3	2	NA	8
	SEG47	0.1	0	2.3	4	3	3	3	3	12
	SEG48	0.1	0	2.81	4	3	3	3	3	12
	SEG49	0	0	2.61	3	3	3	3	NA	9
	SEG50	0	0	1.98	4	3	3	3	3	12

Chapter V

General Discussion

There is ample evidence that evolutionary processes can take place over short periods of time, within a couple, dozens, or one hundred generations; moreover, these rapid changes are often related to human activities (Hendry & Kinnison, 1999; Reznick & Ghalambor, 2001; Carroll *et al.*, 2007). While the effects of land use on species diversity and at the community level have been extensively studied (Socher *et al.*, 2013; Dengler *et al.*, 2014), its relationship to intraspecific variation – the raw material for evolution – has only been shown in studies with smaller sample sizes (van Groenendael, 1986; Pluess, 2013), coarse land-use categories (Warwick & Briggs, 1979) and experimental design not allowing to tease apart land-use effects from other environmental variables (Van Tienderen & van der Toorn, 1991; but see Völler *et al.*, 2013, and 2017).

In this thesis, I investigated the relationship between land use and intraspecific variation in a specific way, supported by a robustly designed study system. The Biodiversity Exploratories with its standardised network of grassland sites across Germany allowed the inclusion of many populations over a broad geographical range, coupled with probably the most precise land-use data coming from a realistic background. I worked with *Plantago lanceolata*, a ubiquitously occurring grassland plant, and combined field and common-garden-derived data that allowed the distinction of environmentally induced and stable intraspecific variation (Bossdorf *et al.*, 2008). In **Chapter II**, I looked at genetic, epigenetic and phenotypic variation in *P. lanceolata*, and their relationships to land use. In **Chapter III**, I then examined phenotypic variation and adaptive potential related to land use in greater detail, and in **Chapter IV**, I looked into possible trade-offs between three particular functional traits in grasslands. Below, I briefly summarise the main results of my work in three areas: (1) patterns of epigenetic variation, (2) patterns of phenotypic means and

variation, and (3) opposing effects of mowing and grazing on several measures of variation.

Patterns of intraspecific epigenetic variation

In **Chapter II**, I focused specifically on two types of intraspecific epigenetic variation: within-population diversities and between-population differentiation. I compared these to genetic and phenotypic variation and related them to land use. The MSAP method used for epigenetic fingerprinting distinguishes between three marker types (MSAP-m, MSAP-h, plus the unmethylated MSAP-n-type), the first two of which correspond respectively to a more stable (CG-context), and a less stable (CHG-context) type of cytosine methylation (Schmitz *et al.*, 2013; Schulz *et al.*, 2013). I found small but significant epigenetic differentiation between the 60 populations, and also significant genetic differentiation based on AFLP markers. The more unstable the markers were, the more differentiation they showed, and the smaller their intrapopulation diversity was. The latter indicated environmental induction, further corroborated by the mostly lower diversities and consistently weaker differentiation in the common environment.

Epigenetic diversity was consistently negatively correlated to mowing, and positively to grazing intensity, except for the lack of relationship with grazing for the less stable MSAP-h-diversities in the common environment. I find three conclusions particularly worth mentioning about my results: i) on other levels of biological organisation, mowing is already known as a strong homogenising factor, while the opposite is true for grazing (see also the third section of the General Discussion), and intraspecific epigenetic variation appears to follow the same pattern; ii) mowing was found in previous studies to be the land-use component with the strongest effects on various response variables (e.g. Völler *et al.*, 2013, 2017; Gossner *et al.*, 2016), and I showed that its effect remains also in the F_1 generation in a common environment in all types of DNA-methylation, while not in the case of grazing; iii) it is the least stable MSAP-h epilocus type (Schmitz *et al.*, 2013; Schulz *et al.*, 2013) that doesn't maintain its relationship to grazing in the common environment.

Phenotypic trait means, variation, and trade-offs

In **Chapter III** I explored the phenotypic data from the common-garden experiment in greater detail, and in **Chapter IV** I reported the results from an additional experiment where I measured three highly relevant, but more derived, functional traits, with a special focus on their interrelationships. Altogether, I measured twelve phenotypic traits, with ten out of them showing differentiation between maternal seed families, and seven between populations. Most importantly, competitive ability expressed as *1-R** (Tilman, 1985) was differentiated on both levels, and to our knowledge, this is the first instance of showing intraspecific variation in *R**, a key functional trait in plant ecology.

In **Chapter III**, I focused on the population means and variation of traits, and their relationships to mowing, fertilisation, and grazing. Some of these relationships were significant, especially between trait means and mowing, but the dataset is noisy in general. The traits measured in **Chapter IV** were not significantly related to any land-use component. One pattern was conspicuous though: across all significant and non-significant land use-trait relationships there was a clear pattern that mowing and grazing had opposite effects on the measured phenotypes, both in terms of their means and their variances. There seem to be two distinct trait syndromes: larger plants with a lower specific leaf area (SLA) are associated with high mowing intensity, and smaller plants with higher SLA are associated with high grazing intensity. This did seem surprising at first look, as higher SLA is usually correlated with higher growth rates and a less conservative metabolism. However, this common knowledge comes from studies comparing species across biomes and with very different growth forms (Wright *et al.*, 2004; Poorter *et al.*, 2009; Díaz *et al.*, 2016), and variation in SLA at the intraspecific level might behave differently (Milla & Reich, 2007; Scheepens *et al.*, 2010). There is literature showing that in the case of grazing lawns the relationship between plant size and SLA is exactly as in our case (McNaughton, 1984; Cingolani *et al.*, 2005). The required conditions for grazing lawns observed by these authors are i) grazing tolerance instead of avoidance, ii) herbivore preference, and iii) high resource availability; which are all fulfilled in our case (see **Chapter III**). Within-population variation, representing genetic diversity in

phenotype, and thus evolutionary potential, was decreased by mowing and increased by grazing, similarly to the findings of **Chapter II**.

Despite the lack of land-use effects on the three functional traits measured in **Chapter IV**, there were significant relationships among these traits. Competitive ability (*1-R**) was positively correlated with clipping tolerance at the population level, but not at the levels of individuals or seed families, indicating that this relationship represents adaptation to habitat, conferring benefits in the same environment, but without a deeper physiological or genetic linkage. Clipping tolerance and the response to a nutrient pulse, on the other hand, showed a negative correlation at the level of individuals and seed families, but not populations. When I further dissected this relationship at the individual level, I found that it remained only in plants from plots with low mowing intensities and disappeared at higher mowing frequencies. This indicates a trade-off because the sites with low mowing intensities are also less fertilised and thus resource-limited, which is a requirement for trade-offs (Agrawal *et al.*, 2010), whereas the sites where the negative correlation does not hold have more resources because of the more frequent mowing and fertilisation.

Contrasting effects of mowing versus grazing

Mowing and grazing are known to be inherently different processes. Mowing is much more homogenising (Gossner *et al.*, 2016), whereas grazing increases habitat heterogeneity in several different ways (Bakker *et al.*, 1984; Adler *et al.*, 2001; Gibson, 2009). Therefore, to promote biodiversity, low-intensity grazing is considered a preferable management technique over mowing, but many previous studies yielded idiosyncratic results about species diversity (Dengler *et al.*, 2014), making it hard to generalise. In my thesis, however, whenever mowing or grazing had significant effects on the measured dependent variables, the patterns were according to the general expectation. Epigenetic (but not genetic) diversity consistently decreased with mowing, both under field and common garden conditions, while it increased with grazing (**Chapter II**). This is in line with other findings from the Biodiversity Exploratories that showed mowing to be the strongest selective force among the main land-use factors (Gossner *et al.*, 2016; Völler *et al.*,

2017). Likewise, phenotypic diversity decreased with mowing and increased with grazing, and many phenotypic traits showed opposite relationships to the two land-use components at the population level (**Chapter III**). In **Chapter IV**, the only significant relationship with land use was the modulation of a trade-off between two functional traits, and indeed, even this proved to be opposing between mowing and grazing. Altogether, mowing and grazing appear to have fundamentally different effects on the intraspecific variation of the studied plant populations, and appear to drive evolution in contrasting directions.

Conclusions and outlook

Plantago lanceolata seems to be a very attractive system for eco-evolutionary studies, as it is a very widespread species and easy to handle in experiments, and this is reflected in the rich evolutionary-ecological literature (Sagar & Harper, 1964; Warwick & Briggs, 1979; Kuiper & Bos, 1992; Laine, 2008) and ongoing projects (eg. www.plantpopnet.com) based on the species. However, as mentioned earlier, it is also a species with high rates of gene flow, heterozygosity, and phenotypic plasticity, and therefore *P. lanceolata* data often have a high signal-to-noise ratio, especially at the spatial scale we have been working on. But exactly this drawback underpins our results: if differentiation and adaptation *are* found in *P. lanceolata*, most probably they are in fact there, and very likely to be found in other plant species as well.

Nevertheless, the work summarised in my thesis could be improved in many ways, although most of these improvements would involve giving up an existing advantage of the project (i.e. trade-offs between precision, realism and generality; Levins, 1966). The precision could be increased by applying higher-resolution epigenomic methods, such as reduced-representation bisulfite sequencing (Paun *et al.*, 2019), or whole genome bisulfite sequencing (Cokus *et al.*, 2008), but the number of samples, especially from the field would have to be decreased (reducing generality). Another way of enhancing the precision could be a different breeding design including more replicates within maternal seed families, and/or working with the F_2 generation to reduce the stochasticity introduced by the random fathers in the F_1 cohort, but this would decrease the number of populations included (and so again

the generality). There could be more species included, or the geographical scale could be extended to enhance the generality of the project, but this would either require decreasing the number of samples (precision) or populations (generality). The realism of this work could be improved by taking more sophisticated phenotypic measurements or measuring fitness directly, that however would sacrifice generality as we would need to reduce e.g. the number of populations. Another way to strengthen the realism would be by having an external field site as a common growing environment, however, this holds the possibility of reducing precision drastically, as it happened in the first attempt of my project, when the common garden plants were completely wiped out by mice.

The particular ‘compromise approach’ of precision, realism and generality employed in my thesis allowed me to shed light on the structure and stability of population epigenetic patterns across a high number of natural populations, to describe significant intraspecific variation in, and trade-offs among several phenotypic traits, including R^* for the first time, and to find support for the contrasting effects of mowing and grazing on phenotypic trait syndromes.

References

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

Agrawal AA, Conner JK, Rasmann S. 2010. Tradeoffs and negative correlations in evolutionary ecology. In: M. A. Bell, W. F. Eanes, D. J. Futuyma, and J. S. Levinton, ed. *Evolution After Darwin: The First 150 Years*. 243–268.

Bakker JP, de Leeuw J, van Wieren SE. 1984. Micro-patterns in grassland vegetation created and sustained by sheep-grazing. *Vegetatio* 55: 153–161.

Bossdorf O, Richards CL, Pigliucci M. 2008. Epigenetics for ecologists. *Ecology Letters* 11: 106–115.

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

Cingolani AM, Posse G, Collantes MB. 2005. Plant functional traits, herbivore selectivity and response to sheep grazing in Patagonian steppe grasslands. *The Journal of Applied Ecology* 42: 50–59.

Cokus SJ, Feng S, Zhang X, Chen Z, Merriman B, Haudenschild CD, Pradhan S, Nelson SF, Pellegrini M, Jacobsen SE. 2008. Shotgun bisulphite sequencing of the *Arabidopsis* genome reveals DNA methylation patterning. *Nature* 452: 215–219.

Dengler J, Janišová M, Török P, Wellstein C. 2014. Biodiversity of Palaearctic grasslands: a synthesis. *Agriculture, Ecosystems & Environment* 182: 1–14.

Díaz S, Kattge J, Cornelissen JHC, Wright IJ, Lavorel S, Dray S, Reu B, Kleyer M, Wirth C, Prentice IC, *et al.* 2016. The global spectrum of plant form and function. *Nature* 529: 167–171.

Gibson DJ. 2009. *Grasses and Grassland Ecology*. Oxford University Press.

Gossner MM, Lewinsohn TM, Kahl T, Grassein F, Boch S, Prati D, Birkhofer K, Renner SC, Sikorski J, Wubet T, *et al.* 2016. Land-use intensification causes multitrophic homogenization of grassland communities. *Nature* 540: 266–269.

van Groenendael JM. 1986. Life history characteristics of two ecotypes of *Plantago lanceolata* L. *Acta Botanica Neerlandica* 35: 71–86.

Hendry AP, Kinnison MT. 1999. Perspective: The pace of modern life: Measuring rates of contemporary microevolution. *Evolution* 53: 1637–1653.

Kuiper PJC, Bos M. 1992. *Plantago: A Multidisciplinary Study*. Springer.

Laine A-L. 2008. Temperature-mediated patterns of local adaptation in a natural plant-pathogen metapopulation. *Ecology Letters* 11: 327–337.

Levins R. 1966. The strategy of model building in population biology. *American Scientist* 54: 421–431.

McNaughton SJ. 1984. Grazing lawns: animals in herds, plant form, and coevolution. *The American Naturalist* 124: 863–886.

Milla R, Reich PB. 2007. The scaling of leaf area and mass: the cost of light interception increases with leaf size. *Proceedings of the Royal Society B — Biological Sciences* 274: 2109–2114.

Paun O, Verhoeven KJF, Richards CL. 2019. Opportunities and limitations of reduced representation bisulfite sequencing in plant ecological epigenomics. *The New Phytologist* 221: 738–742.

Pluess AR. 2013. Meta-analysis reveals microevolution in grassland plant species under contrasting management. *Biodiversity and Conservation* 22: 2375–2400.

Poorter H, Niinemets Ü, Poorter L, Wright IJ, Villar R. 2009. Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *The New Phytologist* 182: 565–588.

Reznick DN, Ghalambor CK. 2001. The population ecology of contemporary adaptations: what empirical studies reveal about the conditions that promote adaptive evolution. *Genetica* 112: 183–198.

Sagar GR, Harper JL. 1964. *Plantago major* L. *P. media* L. and *P. lanceolata* L. *The Journal of Ecology* 52: 189–221.

Scheepens JF, Frei ES, Stöcklin J. 2010. Genotypic and environmental variation in specific leaf area in a widespread Alpine plant after transplantation to different altitudes. *Oecologia* 164: 141–150.

Schmitz RJ, Schultz MD, Urich M a., Nery JR, Pelizzola M, Libiger O, Alix A, McCosh RB, Chen H, Schork NJ, *et al.* 2013. Patterns of population epigenomic diversity. *Nature* 495: 193–198.

Schulz B, Eckstein RL, Durka W. 2013. Scoring and analysis of methylation-sensitive amplification polymorphisms for epigenetic population studies. *Molecular Ecology Resources* 13: 642–653.

Socher SA, Prati D, Boch S, Müller J, Baumbach H, Gockel S, Hemp A, Schöning I, Wells K, Buscot F, *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.

Tilman D. 1985. The resource-ratio hypothesis of plant succession. *The American Naturalist* 125: 827–852.

van Tienderen PH, van der Toorn J. 1991. Genetic differentiation between populations of *Plantago lanceolata*. I. Local adaptation in three contrasting habitats. *The Journal of Ecology* 79: 27–42.

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. *The Journal of Ecology* 105: 1290–1297.

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.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornelissen JHC, Diemer M, *et al.* 2004. The worldwide leaf economics spectrum. *Nature* 428: 821–827.

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Publications

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Richards CL, Alonso C, Becker C, Bossdorf O, Bucher E, Colomé-Tatché M, Durka W, Engelhardt J, **Gaspar B**, Gogol-Döring A, et al. 2017. Ecological plant epigenetics: Evidence from model and non-model species, and the way forward. *Ecology Letters* 20: 1576–1590.

Balázs TK, Błaszkowski J, Chwat G, Góralska A, **Gáspár BK**, Lukács AF, Kovács GM. 2015. Spore-based study of arbuscular mycorrhizal fungi of semiarid sandy areas in Hungary, with *Diversispora jakucsiae* sp. nov. *Mycological progress* 14: 1021.

Blaszkowski J, Chwat G, Kovács GM, **Gáspár B**, Ryszka P, Orlowska E, Pagano MC, Araújo F, Buscot F, Wubet T. 2012. *Septoglomus fuscum* and *S. furcatum*, two new species of arbuscular mycorrhizal fungi (Glomeromycota). *Mycologia* 105: 670–680.

Blaszkowski J, Kovács GM, **Gáspár BK**, Balázs TK, Buscot F, Ryszka P. 2012. The arbuscular mycorrhizal *Paraglomus majewskii* sp. nov. represents a distinct basal lineage in Glomeromycota. *Mycologia* 104: 148–156.

Manuscripts in preparation

Gáspár B, Bossdorf O, Parepa M (2019) Intraspecific variation in land use-related functional traits in *Plantago lanceolata*. (In prep.)

Gáspár B, Durka W, Bossdorf O (2019) Rapid evolution in managed grasslands: different land-use regimes are associated with contrasting phenotypes in *Plantago lanceolata*. (In prep.)

Conference contributions

2018/08 Integrating large-scale genetic, epigenetic and phenotypic data from natural populations of *Plantago lanceolata* to understand the influence of land use on intraspecific diversity and adaptation (**POSTER**) II Joint Congress on Evolutionary Biology, Montpellier, France

2018/05 Trade-offs between growth strategies in managed grasslands: within-species variation in *Plantago lanceolata* (**POSTER**) PopBio, 31th Conference of the Plant Population Biology Section of the Ecological Society of Germany, Austria and Switzerland (GfÖ), Innsbruck, Austria

2017/11 Natural epigenetic diversity in the common grassland plant *Plantago lanceolata*. (**POSTER**) The meeting of Students in Evolution and Ecology (Meeting StEvE), Tübingen, Germany

2017/05 Natural epigenetic diversity in the common grassland plant *Plantago lanceolata*. (**TALK**) PopBio, 30th Conference of the Plant Population Biology Section of the Ecological Society of Germany, Austria and Switzerland (GfÖ), Halle, Germany

2016/09 Phenotypic plasticity of *Plantago lanceolata* in relation to land use. (**TALK**) 46th Annual Meeting of the GfÖ, Marburg, Germany

2016/05 Phenotypic plasticity of *Plantago lanceolata* in relation to land use. (**POSTER**) PopBio, 29th Conference of the Plant Population Biology Section of the Ecological Society of Germany, Austria and Switzerland (GfÖ), Třeboň, Czech Republic