Effects of climate change on plants and ecosystem functioning: Implications for managed temperate grasslands

Doctoral thesis

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Short summary of the thesis

Global climate change poses challenges to plants and ecosystem functioning. Grasslands have become a major study object in experimental biodiversity and climate impact studies. The great majority of the existing studies investigated the effects of climate change on productivity. However, studies on how climate change (such as 1000-year drought, high precipitation variability, seasonal warming, late frost in spring etc.) affects flowering phenology, plant physiology, community composition, legume facilitation, plant nitrogen (N) and soil N status in managed temperate grasslands are lacking. It is known that land management can improve performances of plants and ecosystem functions. Yet, the relative importance and potential of land management in buffering the negative impacts of climate change are largely unknown. In addition, the rain-out shelters used to study the ecological responses to climate change (mainly drought) are often criticized for creating micro-climatological artifacts, which may influence plant responses.

Thus, the main objectives of this thesis were (a) to investigate how selected plants and ecosystems respond to different aspects of climate change (e.g. seasonal warming, precipitation variability, winter rain addition, late frost, heavy rainfall and drought), (b) to investigate three potential land management options to buffer the negative impacts of climate change, and (c) to contribute to the advancing of climate change research by examining whether there are any methodological artifacts in ongoing climate manipulations experiments. To meet these three objectives, responses (mainly related to phenology, productivity, physiology, seedling emergence and N status) of selected plant species, their populations, artificial plant communities as well as a semi-natural managed temperate grassland ecosystem were investigated.

Seasonal (winter/summer) warming advanced flowering phenology and altered biomass production of early vs. late flowering species (manuscript 1). Onset of early flowering temperate grassland species was advanced by winter warming (4.9 days) more than by summer warming (2.3 days), while late flowering species were generally less sensitive to warming in either season. Flowering phenology was largely unaffected by experimental changes in precipitation regimes (manuscript 1). However, high precipitation variability during the growing season altered plant cover of early vs. late flowering species. Ecosystem productivity and legume facilitation increased under heavy rainfall compared to control (manuscript 2). Drought reduced plant physiological activities e.g. lower stomatal conductance, lower effective quantum yield, and lower leaf water potential (manuscript 6).

Drought effects on plants were altered by the presence of legume species (manuscript 2). Under drought, the presence of a legume species enhanced overall biomass production of three neighboring grassland species by 36% compared to the absence of legume. Species-specific legume facilitation effects were also detected: *Arrhenatherum elatius* was facilitated by legume presence

under drought and heavy rainfall, *Plantago lanceolata* was facilitated only under heavy rainfall, and *Holcus lanatus* was facilitated only under control conditions. Positive effects of legume presence found under control also persisted under drought for plant and soil N.

European populations/provenances of grass species differed in plant N status under drought. Yet, populations from the wetter sites did not perform worse than presumably drought-adapted populations, indicating no evidence of local adaptation (manuscript 3). Variation in within-species responses was as high as variation in among-species responses under drought and late frost (manuscript 5). Within-species variation during the early life stages of *Verbascum thapsus* populations (a global plant invader) was detected as different germination and seedling emergence rates under the representative climates of seven biomes (manuscript 4). Furthermore, plant N status was altered by rewetting and harvest delay after drought (manuscript 3). Harvest delay after rewetting could not compensate the negative effects of drought on biomass production, but increased plant N concentration and N content.

A detailed quantification of micro-climatological artifacts showed that the strength of drought manipulation using the rain-out shelter technique was dependent on ambient weather conditions (manuscript 6). Plant responses were highly correlated to ambient micro-climate conditions. Therefore, relating drought responses to ambient micro-climatological parameters such as air temperature and vapor pressure deficit can facilitate meaningful interpretation and comparison of studies and of different responses of experimental droughts between years within single studies. Furthermore, rain-out shelters altered temperature and reduced radiation inside the shelter. However, these micro-climatological artifacts had no significant effects on growth responses of grassland plants. Thus, fixed rainout shelters remain a useful tool for ecological drought manipulation experiments.

In summary, the present thesis provides evidence on how climate change affects selected plant species and ecosystem functions in managed temperate grasslands. The findings of this thesis have practical implications for grassland ecosystem management in the face of climate change. For instance, negative drought effects can be minimized by legume presence and by rewetting combined with harvest delay. Results show strong differences in population-specific responses to extreme climatic conditions. However, climatic origin of populations cannot predict these response variations. Therefore, increasing within-species diversity (or population mixtures) may help maintain plant productivity and N nutrition in the face of climate change.

Kurze Zusammenfassung der Doktorarbeit

Titel: Auswirkungen des Klimawandels auf Pflanzen und Ökosystemfunktionen: Implikationen für Wirtschaftsgrünland der gemäßigten Breiten

Zusammenfassung

Der globale Wandel stellt Pflanzen sowie Ökosysteme und die damit verbundene Bereitstellung von Ökosystemdienstleistungen vor eine Herausforderung. Grasländer haben sich innerhalb der Disziplinen Biodiversitätsforschung, sowie Klimawandeleffektforschung als eines der Hauptforschungsobjekte herausgestellt. Der Großteil der bestehenden Studien beschäftigt sich mit den Auswirkungen des Klimawandels auf die Produktivität solcher Grasländer. Jedoch sind Studien in denen der Frage nach den Auswirkungen des Klimawandels (z.B. Dürre, hohe Niederschlagsvariation, Bodenerwärmung, Spätfrost im Frühjahr etc.) auf differenziertere ökologische Parameter, wie z.B. Phänologie, Physiologie, Artzusammensetzung, Wachstumsförderung durch Anwesenheit von Leguminosen, Pflanzen verfügbarer Stickstoff, sowie Pflanzenstickstoffgehalte nachgegangen wurde, unterrepräsentiert. Es ist bekannt, dass durch Bewirtschaftungsmaßnahmen die Wuchsleistung von Pflanzenbeständen, sowie die Bereitstellung von Ökosystemdienstleistungen verbessert werden kann. Das Potenzial von Grünland-Bewirtschaftung im Hinblick auf eine abmildernde Wirkung der prognostizieren, negativen ökologischen Auswirkungen des Klimawandels sind allerdings größtenteils unbekannt. Zudem sind die Methoden, die genutzt werden um zu untersuchen inwieweit sich der Klimawandel (im engeren Sinne Dürre) auf Ökosysteme auswirken kann, durch ihre ungewollten mikro-klimatischen Effekte (Artefakte) umstritten.

Die Hauptzielsetzungen der vorliegenden Dissertationsschrift sind (a) zu untersuchen wie ausgewählte Pflanzen und Ökosysteme auf unterschiedliche Aspekte des Klimawandels (z.B. Bodenerwärmung, Niederschlagsvariabilität, Winter-Starkregen, Spätfrost, Starkregen und Dürre) reagieren, (b) herauszufinden inwieweit sich drei ausgewählte Bewirtschaftungsmaßnahmen dazu eignen die Auswirkungen des Klimawandels zu kompensieren/abzumildern, und (c) die Forschungsdisziplin der Klimawandeleffektforschung durch die fachliche Reflexion angewandter experimenteller Methoden voranzutreiben. Zu diesem Zwecke wurden die Reaktionen (im Wesentlichen: Phänologie, Produktivität, Physiologie, Keimungsraten und Stickstoffgehalte) ausgewählter Pflanzenarten, ihrer Populationen, sowie von künstlichen Pflanzengemeinschaften und einem bewirtschafteten Grünlandökosystem untersucht.

Saisonale Bodenerwärmung (im Winter/Sommer) führte zu einem früheren Blühbeginn und veränderter Produktivität von ansonsten Früh- bzw. Spätblühenden Arten (Manuskript 1). Das Einsetzen der Blüte bei frühblühenden Graslandarten der gemäßigten Breiten wurde infolge von Bodenerwärmung im Winter (4,9 Tage früher) stärker begünstigt als bei der Erwärmung im

Sommer (2,3 Tage), während spät-blühende Arten generell weniger anfällig gegenüber den experimentellen Erwärmungen waren. Infolge experimenteller Veränderungen des Niederschlagsregimes zeigten sich keinerlei Verschiebungen der Phänologie (Manuskript 1). Dennoch zeigte sich der Einfluss von experimentell variierten Niederschlagsverhältnissen in der Wachstumsperiode in einer Veränderung des Bedeckungsgrades von Spät- bzw. Frühblühern. Produktivität und Wachstumsförderung durch Anwesenheit von Leguminosen nahmen unter simulierten Starkregenbedingungen zu im Vergleich zu den Kontrollbedingungen (Manuskript 2). Dürre verringerte pflanzenphysiologische Parameter wie stomatäre Leitfähigkeit, effektive Quantenausbeute und Wasserpotential der Blätter (Manuskript 6).

Dürreeffekte auf Pflanze wurden durch die Anwesenheit von Leguminosen beeinflusst (Manuskript 1). Unter Dürrebedingungen hatte die Anwesenheit von Leguminosen die Gesamtbiomasseproduktion von drei benachbarten Graslandarten um 36% erhöht. Darüber hinaus wurde artspezifische Begünstigung anderer Pflanzenarten durch Leguminosen nachgewiesen: Glatthafer (*Arrhenatherum elatius*) wurde während Dürre- und Starkregenereignissen durch Leguminosen-Präsenz begünstigt, während Spitzlattich (*Plantago lanceolata*) nur während Starkregen und Wolliges Honiggras (*Holcus lanatus*) nur unter Kontrollbedingungen begünstigt wurden. Die innerhalb der Kontrollbedingungen durch die Anwesenheit von Leguminosen hervorgerufenen positiven Effekte auf N-Gehalte in Boden und Pflanze bestanden auch während des Einflusses der Dürre fort.

Europäische Graspopulationen/-provenienzen verhielten sich unterschiedlich unter Dürrebedingungen. Allerdings verhielten sich Populationen aus humiden Gebieten nicht schlechter als aus semi-ariden Gebieten, was darauf hinweist, dass es keine lokale Anpassung gibt (Manuskript 3). Sowohl unter Dürre- als auch unter Spätfrostbedingungen war die Variation innerhalb von Arten in den meisten Fällen so hoch wie zwischen den Arten (Manuskript 4). Es konnte (für sieben unterschiedliche, experimentell nachgestellte Klimabedingungen) gezeigt werden, dass für die Art *Verbascum thapsus* (eine global invasive Pflanzenart) innerartliche Variation (d.h. unterschiedliche Keimungs- und Keimlingsetablierungsraten) hauptsächlich während früher Lebensabschnitte vorkommt (Manuskript 4). Des Weiteren wurden Pflanzenstickstoffgehalte durch Wiederbefeuchtung und Ernteverzögerung nach der Dürre verändert (Manuskript 3). Eine Ernteverzögerung nach der Wiederbefeuchtung konnte aber nicht die negativen Dürreeffekte auf die Biomasseproduktion kompensieren, führte aber zu erhöhten Pflanzenstickstoffkonzentrationen und -gehalten.

Eine detaillierte Charakterisierung der mit der Nutzung von Überdachungen verbundenen mikroklimatischen Effekte zeigte, dass die Rolle von methodischen Artefakten bei diesem experimentellen Ansatz stark von den jeweils vorherrschenden Wetter-Bedingungen abhängig ist (Manuskript 6). Die Reaktion der untersuchten Pflanzen war stark mit den Wetterverhältnissen der

Umgebungsluft (unter Kontrollbedingungen) korreliert. Die Betrachtung von Dürreeffekten im Zusammenhang mit mikro-klimatischen Parametern wie Lufttemperatur und Dampfdruckdefizit (VPD) kann hiernach die Interpretation und den Vergleich unterschiedlicher Studien sowie Unterschiede in verschiedenen Jahren einer Studie erleichtern. Weiterhin konnte gezeigt werden, dass durch die verwendeten Überdachungen, sowohl Temperatur- als auch Einstrahlungsverhältnisse verändert wurden. Jedoch hatten die mit den Überdachungen verbundenen mikro-klimatischen Veränderungen (Artefakte) keine signifikanten Effekte auf die Trockenheits-induzierte Reaktion der untersuchten Pflanzen. Demnach handelt es sich bei diesen fest-installierten Überdachungen nach wie vor um eine nutzbare Methode in der experimentellen Ökologie.

Die hier vorgelegte Arbeit liefert wichtige Erkenntnisse zu den Auswirkungen des Klimawandels auf die ökologische Funktionsweise ausgewählter Pflanzenarten und des Gesamt-Systems bewirtschafteter Grünländer, die für eine nachhaltige Bewirtschaftung dieser Ökosysteme von fachlicher Relevanz sind. So können beispielsweise Trockenheits-induzierte Effekte durch die Anwesenheit von Leguminosen und durch Bewässerung in Kombination mit einer verzögerten Ernte reduziert werden. Die im Zuge dieser Arbeit präsentierten Ergebnisse zeigen zudem starke Unterschiede in der Reaktion unterschiedlicher Populationen einer Art auf klimatische Extrembedingungen. Dennoch konnte die (klimatische) Herkunft der untersuchten Populationen keine Rückschlüsse auf die Art und Richtung der gezeigten Reaktion geben. Demnach kann das Mischen unterschiedlicher Populationen oder eine generell hohe, intra-spezifische Diversität als geeignete Maßnahme empfohlen werden, um in Angesicht des aktuellen Klimawandels die Produktivität und Nährstoff- (Stickstoff-) Verfügbarkeit zu sichern.

Thesis outline

My thesis starts with a general background of different aspects of ongoing climate change and introduces extreme climatic events that are subject to this work. After that, I perform a review on (a) impacts of climate change mainly in managed temperate grasslands, (b) relative importance of land management in buffering negative impacts of climate change, and (c) micro-climate artifacts in climate manipulation experiments to identify research gaps and to formulate my thesis objectives. Subsequently, the motivation, key results and synthesis of the six manuscripts are presented to provide an integrated picture of my thesis. In that chapter, I show impacts of investigated climate manipulations on plants and ecosystem functions of managed temperate grasslands, recommend three potential management options to mitigate climate change effects, and suggest additional measurements to make experimental climate impact studies comparable. Several future research challenges that have emerged from this thesis are discussed in the following chapter. The six manuscripts are finally presented after the declaration of my own contribution, along with their current status (see **figure 1** for graphical outline of the thesis). Explanations and definitions of specific terms that are not explained in the text are provided separately in **Box 1**.

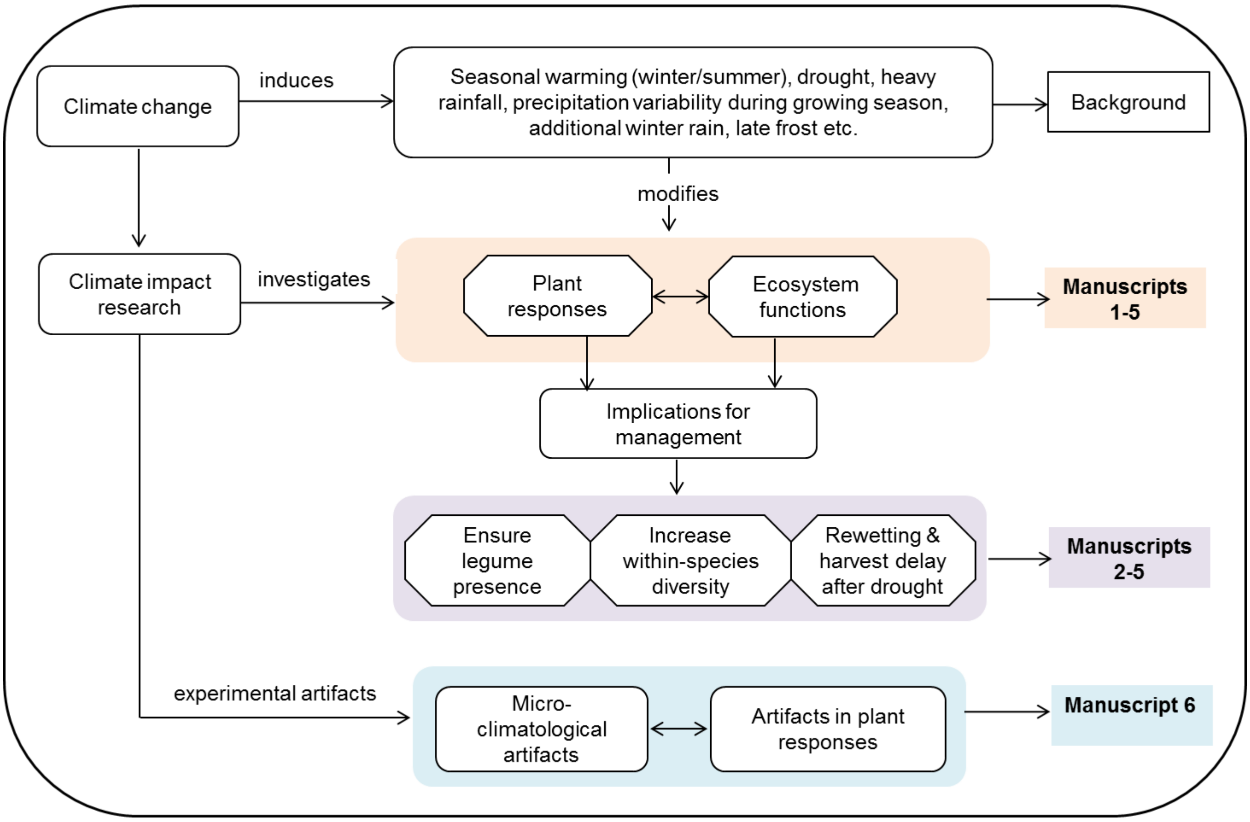

Figure 1: Graphical outline of the thesis, showing the concept of each manuscript.

Box 1: Definitions and explanations of terms as used in this thesis

Chronic drought: Partial withdrawal of natural precipitation over longer periods of time. Chronic drought is experimentally realized with translucent rain-out strips by partial reduction of precipitation e.g. 30%, 50%, or 80% etc.

Climatic extreme: It is an event or episode or occurrence in which a period of statistical climate extremity alters ecosystem functions outside the bounds of what is considered typical or normal variability. In the thesis, extremeness of weather treatments is determined by statistical extremity with respect to a historical reference period (extreme value theory), independent of its effects on vegetation. Growing seasons (March–September) of 1961–2000 in Central Europe (Germany) are used as a reference period. Gumbel I distributions are fitted to the annual extremes, and 100 or 1000-year recurrent events are calculated.

Legume facilitation: A process by which a plant that does not fix atmospheric nitrogen profits from the presence of nitrogen-fixing legume plant. Legume facilitation increases the effectiveness/performances of other co-existing non-legume plants.

Local adaptation: It is a process that enhances the fitness of an organism in their local environment by bringing a population/ecotype/provenance closer to their fitness maxima. Adaptation to a local environment can lead to an evolution of one population that differs morphologically/ physiologically /genetically from other populations of the same species. Within a species' range, individual populations experience site-specific environmental history. For instance, if a plant population was continuously confronted with extremely dry conditions, selection processes will select for the best adapted members.

Population/Ecotype/provenance: A group of organisms distinguished by morphological and physiological characters which are determined by genes restricted to the geographical regions in which the organisms occur. The biological units occupy a more or less well-defined and continuous geographic area and can interbreed with each other.

Pulse drought: Complete reduction of natural precipitation for defined, brief periods of time. Pulsed drought is experimentally simulated under rain-out shelters by a complete elimination of precipitation (via covering of plots).

Resistance: The degree of change after disturbance (here, extreme climatic conditions). It is a property of a populations, species or communities to remain unaffected in the context of any environmental stress or perturbation or disturbance. Resistance is usually calculated as the difference of a particular response measure between disturbed and undisturbed conditions and reflects the extent to which the mean of an ecosystem property changes after a single stress event. Resistance is one of the major aspects of ecological stability.

Resilience: It is the capacity of populations, species or communities to recover quickly after a stress event or perturbation or disturbance. Resilience is the rate of return of an ecosystem after disturbance to its pre-disturbance level at a given time.

1 Background

1.1Global climate change: causes and consequences

Climate change is usually defined as changes in the mean distribution and/or the variability of climate properties that continues for an extended period of time, typically decades to millions of years (IPCC 2012).

Natural processes (e.g. changes in solar radiation, volcanic eruptions, and deviations in the Earth's orbit around the sun) can cause climate change. However, human influences such as fossil fuel burning, land use change and in particular the emission of greenhouse gases are recognized as dominant causes of ongoing global climate change, often referred to as "global warming". The recent report of IPCC (2013) states that atmospheric concentration of anthropogenic greenhouse gases like carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) have increased by 40%, 150% and 20%, respectively, compared to pre-industrial levels. If the current trend of emission continues, the atmospheric concentration of carbon dioxide is expected to double within the next few decades.

The increase in anthropogenic greenhouse gases have high contribution to an increase in global mean surface (both land and ocean) temperature by 0.85°C over the period 1880 to 2012 (IPCC 2013). In the last 2000 years, the 1990 to 2010 was the warmest decade in the Northern Hemisphere (Moberg et al. 2005). The years 1998, 2002 and 2003 were the warmest years in the last one thousand years (Moberg et al. 2005; Ciais et al. 2005). Besides further gradual warming, modifications in seasonality are expected (Hegerl et al. 2011a; Min et al. 2011; IPCC 2013). More extreme weather regimes due to increased climatic variability are also expected (Hegerl et al. 2011b), especially in temperate regions (IPCC 2012). In Central Europe, temperature is likely to increase more rapidly in winter than in summer (Jacob 2009; Kreyling and Henry 2011; Andrade et al. 2012; Vautard et al. 2013; Vautard et al. 2014). In Germany, mean temperature has increased by 1°C over the period 1901-2000, with a pronounced temperature increase during the winter season (Zebisch et al. 2005). Continued emission of greenhouse gases will further enhance global warming. Future climate projection shows global mean surface temperature at the end of 21^{st} century relative to 1850-1900 will be 1.5°C higher (Scenario RCP 4.5, 6.0 and 8.5) (IPCC 2013). In Europe, the regional increases in temperature will be higher than that of the global increase, which is likely to exceed 2.6°C at the end of 21^{st} century (EUR 2009).

Mean total precipitation has also increased 0.5 to 1% per decade during the 20th century over most land areas, mainly over the mid-latitude land areas of the Northern hemisphere, while it declined in the subtropical latitudes (EUR 2009; IPCC 2013). With some regional exceptions, the difference in precipitation between wet and dry seasons and between wet and dry areas will also increase over the 21st century (IPCC 2013). Annual precipitation sums are expected to remain unaffected in Europe, but the intra-annual precipitation variation may increase (Jacob 2009; Kotlarski et al. 2014). In this region, precipitation is likely to decrease in summer and increase in winter (Zolina 2012; Kundzewicz et al. 2013).

1.2 Extreme weather events

Changes in mean, variance, or distribution of climatic properties can lead to changes in the timing, duration, frequency and intensity of extreme climatic events (Meehl et al. 2000; Easterling et al. 2000; IPCC 2012). An increase in climatic extremes was recorded in recent years (Coumou and Rahmstorf 2012). This increasing trend was detected in all parts of the world (Alexander et al. 2006; Coumou and Rahmstorf 2012; IPCC 2012). Besides an increasing likelihood for temperature extremes (e.g. heat waves), modified precipitation regimes are also expected. This involves changes in annual precipitation, seasonality, intensity and variability of precipitation, and the frequency and duration of drought periods (IPCC 2013). These changes can also result in increased intra-annual climatic variability. Increased climatic variability may also lead to prolonged periods of extremely wet or dry conditions due to stable cyclone or anticyclone positions (Francis and Vavrus 2012; Petoukhov et al. 2013). More severe extreme climatic conditions are anticipated as a consequence of ongoing global climate change in the near future (Meehl et al. 2000; Easterling et al. 2000; IPCC 2012). Even in areas where long-term average precipitation is increasing, periods of drought and higher variability in soil moisture are expected (IPCC 2012; IPCC 2013). Temperature and precipitation-related extreme events, their trends, observed changes, human contribution to these observed changes and likelihood of further changes are given in the table 1.

The frequency of extreme climatic events has increased in Europe as well (Ciais et al. 2005; EUR 2009; Kundzewicz et al. 2013). An increasing number of heat waves, especially more extremely hot days, has been reported in this region during the 20th century (Alexander et al. 2006; Beniston et al. 2007; Kürbis et al. 2009; IPCC 2012). On the other hand, the number of frost days has slightly decreased and the probability of late frosts in early spring is gradually increasing (Rigby and Porporato 2008; Kreyling and Henry 2011). At the same time, increasing number of extreme droughts and heavy rainfall events has also been observed in Europe (Ciais et al. 2005; Francis and Vavrus 2012; Kundzewicz et al. 2013; Petoukhov et al. 2013). Future climate projections indicate even more extreme climatic conditions in Europe (Christensen and Christensen 2003; Semmler and Jacob 2004; Kundzewicz et al. 2006; IPCC 2012).

Table 1. Climatic extremes - phenomenons, trends, observed changes, human contributions and likelihoods of future changes[a].

Phenomenon of climate extremes	**Direction of trend**	**Changes observed since 1950**	**Human contribution to observed changes**	**Likelihood[b] of further changes in late 21st century**
Temperature extremes				
Cold days and night	Decreases in frequency	*Very likely*	*Very likely*	*Virtually certain*
Hot days and nights	Increases in frequency	*Very likely*	*Very likely*	*Virtually certain*
Warm spells/heat waves	Increases in frequency and/or duration	*Likely*	*Likely*[c]	*Very likely*
Precipitation extremes				
Heavy precipitation	Increases in frequency, intensity, and/or amount	*Likely*[d]	*More likely than not*	*Very likely*
Drought	Increases in intensity, and/or duration	*Likely*[e]	*More likely than not*	*Likely*[f]

Note:

[a] table modified from IPCC 2013, Table SMP.1.

[b] probability of likelihood scale: *Virtually certain*, 99-100%; *Very likely*, 90-100%; *Likely*, 66-100%; *More likely than not,* >50-100%.

[c] based on available case studies. It is likely that human influence has more than doubled that probability of occurrence of some observed heat waves in some locations.

[d] medium confidence in most countries, *likely* increases in either the frequency or intensity in North America and Europe with some seasonal and/or regional variation.

[e] *likely* increased in the Mediterranean and West Africa, and *likely* decreased in central North America and north-west Australia.

[f] *likely* in dry regions by the end of this century under the RCP8.5 scenario.

1.3 Impacts of climate change and extreme weather events on grassland ecosystems

Current knowledge regarding the effects of warming as well as extreme weather events such as severe drought, heavy rainfall, and late frost on plants and ecosystem functions of grasslands are briefly described below:

1.3.1 Plant and ecosystem responses to warming

Warmer temperatures strongly affect ecosystem productivity and N cycling. Warming generally enhances plant growth, soil N status, soil respiration and decomposition (Walter et al. 2013; Thébault et al. 2014). Warming advances flowering phenology, especially in temperate grassland ecosystems (Menzel and Fabian 1999; Menzel et al. 2006; Hovenden et al. 2008; Bloor et al. 2010; Cornelius et al. 2014). Most phenological studies suggest flowering phenology to be highly sensitive to the average monthly temperature of the preceding two months and the month of flowering onset (Chmielewski and Rötzer 2001; Menzel et al. 2006; Nagy et al. 2013). An advancement in phenology due to warming has impacts on the number of flowers, seed set, timing of seed ripening and seed dispersal (Santandreu and Lloret 1999; Saavedra et al. 2003). Earlier flowering also influences many other plant activities, like leaf expansion, root growth, and nutrient uptake (McKane et al. 1990), which can play important roles in niche differentiation among coexisting species. Warming can reduce plant reproductive fitness by the phenological mismatch between plant and pollinators (Liu et al. 2011). The great majority of these phenological studies are done solely under gradual warming. However, the general knowledge on how flowering phenology responds to winter vs summer warming is missing.

1.3.2 Plant and ecosystem responses to drought

Drought is one of the main stresses anticipated with climate change. It is evident that extreme drought substantially alters ecosystem functions (Breshears et al. 2005; Jentsch et al. 2011; Grant et al. 2014). Drought events have been shown to reduce above-ground plant productivity (Kahmen et al. 2005), increase tissue die back (Kreyling et al. 2008c), shift flowering phenology (Jentsch et al. 2009), reduce ecosystem respiration and photosynthesis (De Boeck et al. 2011). Low soil moisture reduces soil N availability by lowering microbial activity, N mineralization and nutrient diffusion in soils (Voroney 2007; Lambers et al. 2008; Larsen et al. 2011). Drought also reduces total plant N uptake by decreasing transpiration and physical transport in plants (Sardans et al. 2008; Rouphael et al. 2012; Sanaullah et al. 2012; He and Dijkstra 2014). However, drought events can enhance below-ground plant productivity (Kahmen et al. 2005), increase short term microbial biomass (Hartmann et al. 2013) and soil organic matter (Lamarque et al. 2013).

In addition to these negative impacts of drought, it is the modification of nutrient cycling, plant-plant interactions, plant physiological activities and phenological shifts caused by enhanced moisture variability that is of interest in managed ecosystems such as grasslands. Even though grassland community productivity can be strongly resistant to drought (Jentsch et al. 2011, Isbell et al. 2015), legume facilitation, plant physiological activities, phenological responses and nutrient status in plants and soil can be affected.

1.3.3 Plant and ecosystem responses to heavy rainfall

Studies on plant and ecosystem responses to heavy rainfall events are scarce in managed temperate grasslands. Generally, both single plant and ecosystem functions benefit from heavy rainfall, until or unless there waterlogged condition occur. Heavy rain increases aboveground (171%) and belowground (78%) biomass production in a semi-arid grassland (Gao et al. 2011), and increases plant richness of a grassland in Mediterranean climate (Zavaleta et al. 2003b). Kardol et al. (2010) showed that increased precipitation (25 mm/week) explained most of the variation in plant community composition and caused a shift in the proportional biomass among the dominant species (*Lespedeza cuneate*), which resulted in lower community evenness compared to dry

(2mm/week) conditions in an old-field ecosystem. However, in an experimental temperate grassland, overall productivity remained stable in the face of heavy rainfall compared to the control (Kreyling et al. 2008c). In addition, heavy rainfall had no significant effects on the flowering date of temperate grassland plant species (Jentsch et al. 2009).

1.3.4 Plant and ecosystem responses to frost

It is suggested that plants avoid frost stress by completing their life cycle within the growing season (generally frost-free period) or by dormancy (Janská et al. 2010). Very few studies examined the effects of late frost on grassland species (Hare 1995). A study showed that late frost decreases productivity of grasses on average by 20% (Kreyling et al. 2012). Local adaptation of European populations of abundant grass species of temperate grassland has also been detected under late frost (Kreyling et al. 2012). Due to the advanced phenological shift under year-round warming, an earlier exposure of sensitive plant tissue and simultaneously no shifts in the timing of spring frost events, the negative impacts of frost events may increase in grassland ecosystems.

1.4 The importance of land management in grasslands under a changing climate

I have conducted a systematic literature survey to identify the potential land management scenarios that can help maintain grassland ecosystem functioning in a changing climate (see figure 2 for search results). In total, 36 studies were identified (from n=312) that investigated management and climate manipulations together in grasslands (Figure 2a). Within these studies, effects of 10 potential management scenarios (N fertilization, mowing frequency, grazing intensity/frequency, species diversity, species composition, plant/legume facilitation, within-species diversity, rewetting after drought, using grass hybrid, soil C addition/mulching) were investigated under different climate conditions (Figure 2b and Table 2). The combined effects of land management and climate change are also documented in the table 2 in details. In addition, a short summary of these combined effects is provided below:

N fertilization can slightly increase fodder quality (Lamarque et al. 2013), buffer biomass reduction (Hartmann and Niklaus 2012), and increase plant cover (Buckland et al. 2001) under drought (see details in Table 2). The positive effects of fertilization on biomass production increase under higher precipitation, warming and even after modified fire disturbance (Henry et al. 2006; Alhamad et al. 2012; Thébault et al. 2014; Boschma et al. 2015). Under increased precipitation, N fertilization can also increase the dominance of abundant species (Harpole et al. 2007). Under CO_2, warming and increased precipitation, the diversity of forbs abundant species may also become more abundant due to N deposition (Zavaleta et al. 2003a). However, N fertilization has been shown to affect flowering phenology, soil organic matter and soil N mineralization under drought (Lamarque et al. 2013).

Mowing frequency alters biomass production and plant diversity under drought. Bütof et al. (2012) found that under drought and warming annual net primary production (ANPP) increased by 1-time mowing + fertilization compared to 2-3 times mowing + fertilization. It is also found that mowing under drought increase plant diversity in mesic grasslands but decrease plant diversity in xeric grasslands compared to non-mowed plots (Maalouf et al. 2012). Vogel et al. (2012) suggested that both resistance and resilience of an experimental temperate grassland to drought are strongly dependent on intensity of mowing and partly on species diversity. The low canopy height and density after mowing can enhance soil moisture loss through increased soil surface evaporation caused by increased wind speed at the ground level (Zhongmin et al. 2009; Vogel et al. 2012). Species-rich grassland communities have higher evapotranspiration and water use efficiency compared to low diverse grassland community (De Boeck et al. 2006). Extreme drought events can further reduce soil moisture in frequently mown species-rich grasslands. Therefore, low mowing frequency is more important for high resistance of grasslands against drought. In addition, mowing can increase soil N content under drought (Fuchslueger et al. 2014; Dusseux et al. 2015). More frequent cutting strongly stimulates decomposition under warming, but this stimulating effect is absent under extreme precipitation variability, including drought (Walter et al. 2013).

Intermediate grazing compared to high and no grazing increases the diversity of native plants irrespective of climate treatments. Grazing enhances the negative drought effects on plant cover and biomass (Loeser et al. 2007). These negative drought effects can be buffered by species diversity (Suttle et al. 2007; Craine et al. 2012). Species composition is also reported to strongly influence biomass production and species richness in a changing climate and overturning direct precipitation effects within five years in a grassland ecosystem (Suttle et al. 2007). This is because effects of seasonal changes in precipitation are species-specific, and when precipitation regimes persist across several years, feedbacks and species interactions begin to dominate autecological responses to water availability and change community trajectories. The selection of within-species populations which are adapted to extreme weather conditions could be an alternative for maintaining ecosystem functioning in grasslands (Beierkuhnlein et al. 2011; Kreyling et al. 2012).

Furthermore, legume facilitation can increase plant community biomass and stress resistance under both drought and heavy rainfall (Walter et al. 2015, **Manuscript 2**). In addition, rewetting after drought increases soil N status in an alpine grassland ecosystem (Fuchslueger et al. 2014). Rewetting after drought also increases subsurface soil microbial biomass, soil microbial activity and soil N mineralization in a California grassland soils (Xiang et al. 2008).

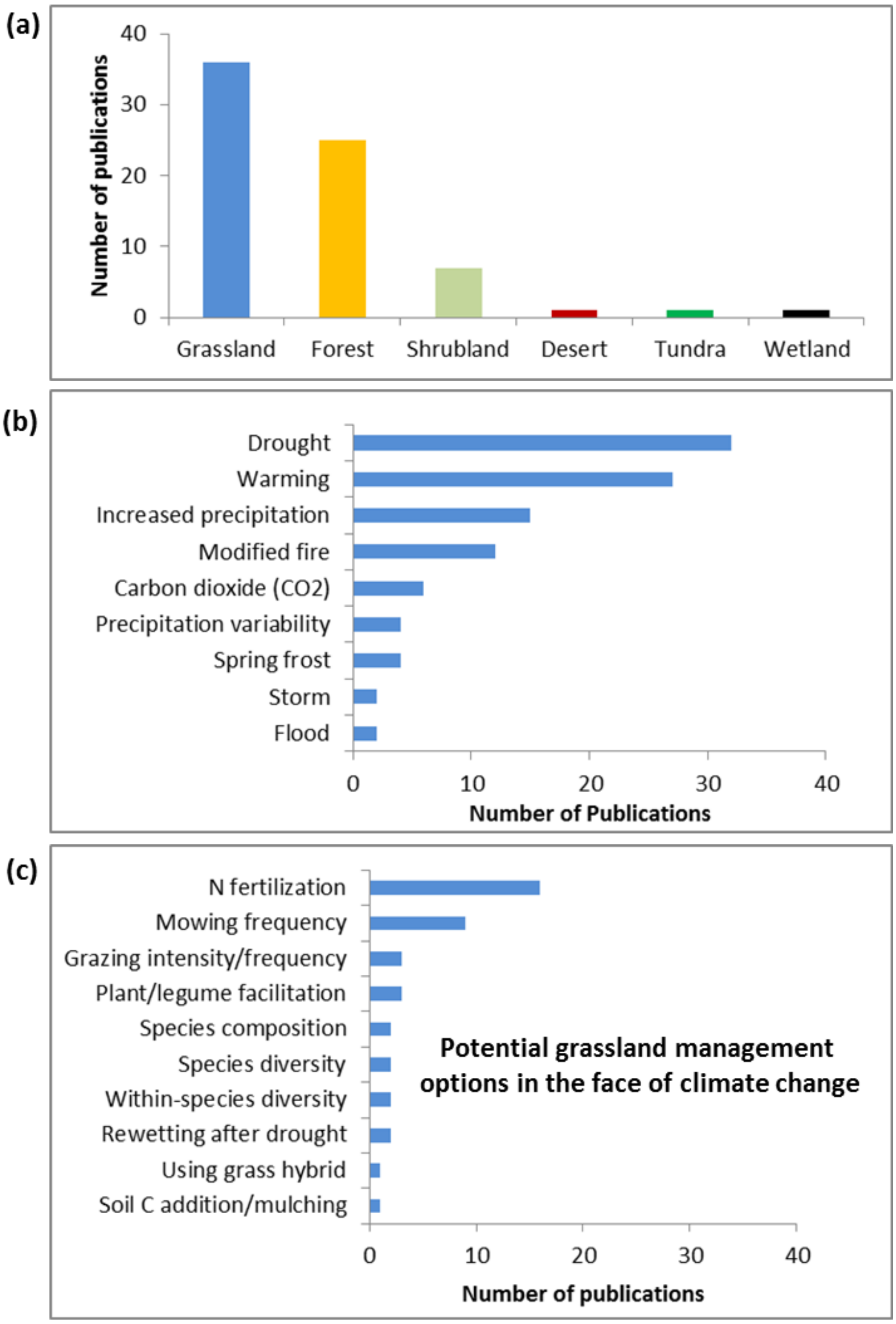

Figure 2: Research on land management options studied in climate change manipulation experiments based on publications found in the ISI Web of Science (a) number of publications that investigated interaction of land managements and climate treatments across ecosystems (n=71, total yield n=312, 241 studies did not study interactions between land management and climate manipulations), (b) number of publications (n=104, including multiple climate treatments in the same study) which investigated different climate manipulations and (c) number of publications (n=41, including multiple management treatments within the same study) which studied the relative importance of land management under different climate treatments only in grassland ecosystems. Search criteria: ISI Web of Science search in August 2015 with the search string: In Title-(Climate* or weather OR drought OR rain* OR frost OR heat* OR warm* OR Cold* OR flood)

AND (Plant OR Vegetation OR Forest OR Grass* OR Heath* OR shrub*) AND In Topic: (Management* AND Climate* AND Experiment*) and the additional condition that the interactions between management and climate treatments were studied in the same manuscript. Search yield n=308 peer-reviewed manuscripts, 4 additional manuscripts (EVENT experiment, Bayreuth) were added to the 308 manuscripts, summing up to 312 studies in total.

The above-mentioned findings indicate that along with N fertilization, modifying mowing frequency, increasing legume presence, increasing within- and among-species diversity have the potential to buffer the negative effects of extreme climatic events such as drought on plant performance and ecosystem functioning. As increased precipitation is beneficial for plants, it is also expected that rewetting can overturn the negative drought effects in many ecosystems.

Table 2: Effects of potential land managements on grassland responses in a changing climate - a review. The peer-reviewed manuscripts that investigated both potential land management options and climate treatments in grasslands are presented here (see figure 2 for ISI Web of Science search details).

Management options	**Climate treatments**	**Ecosystem, location**	**Key findings**	**Source**
Fertilization (manure), mowing and grazing (yes or no)	Drought	Alpine grassland, France	Fertilization and mowing slightly increases crude protein content, and minimizes the direct effects of drought on biomass production and plant diversity. Combined effects of climate and land management (fertilization and mowing) have no effects on flowering onset, litter mass, nitrogen mineralization, soil organic matter, and nitrate retention.	(Lamarque et al. 2013)
N enrichment	Drought	Temperate grassland, Poland	N-deposition has limited effects on plant biomass production and no interactive effects on vegetation biomass or N:P stoichiometry under drought.	(van Dijk et al. 2012)
N fertilization	Drought	Temperate grassland, Switzerland	Fertilization reduces atmospheric methane (CH_4) assimilation in the top soil under drought, presumably because NH_4^+ from fertilizers is not removed by plant uptake and nitrification.	(Stiehl-Braun et al. 2011)
N fertilization	Drought, winter warming	Calcareous grassland, UK	Fertilization triggers species-specific responses to drought and warming. Plant cover of *Holcus lanatus* increased by the combined effects of fertilization and climate treatments. Distribution of *Arrhenatherum elatius* and *Dactylis glomerata* depends on fertilization.	(Buckland et al. 2001)
N fertilization	Drought	Grassland, Switzerland	Fertilization buffers negative drought effects on ANPP.	(Hartmann and Niklaus 2012)
N fertilization	Drought, winter warming	Calcareous grassland, UK	Fertilization decreases seed bank of native species and increase seed bank of non-native species irrespective of climate manipulations.	(Akinola et al. 1998)
N fertilization	Flood, warming	Grassland over many continents	Fertilization increases shoot biomass, root biomass, soil respiration under flood and increase shoot biomass under warming.	(Thébault et al. 2014)
N fertilization	Heavy rainfall	Single grass species, Australia	Fertilization increases ANPP under increased precipitation in summer.	(Boschma et al. 2015)
N fertilization	Precipitation	Semiarid grassland, Mongolia	Fertilization increases ANPP but decreases root to shoot ratio under increased precipitation.	(Gao et al. 2011)

Management options	Climate treatments	Ecosystem, location	Key findings	Source
N fertilization	Precipitation	Grassland, California	Precipitation and fertilization in combination lead to increased dominance of the two most abundant grass species, while N addition, regardless of water availability, decreases species diversity.	(Harpole et al. 2007)
N fertilization	Precipitation, CO_2, Fire	Annual grassland, California	Fertilization increases biomass production under fire and buffers suppressive effects of CO_2. N fertilization alone increases tissue nitrogen–phosphorus ratio.	(Henry et al. 2006)
N deposition	Precipitation, CO_2, warming	Annual grassland, California	N deposition decreases diversity of forbs under the interaction of precipitation, CO_2 and warming .	(Zavaleta et al. 2003a)
N fertilization	Warming	Temperate grassland, Canada	Fertilization doubles the biomass production under warming, N addition alone increases normalized difference vegetation index (NDVI).	(Hutchison and Henry 2010)
N fertilization	Fire	Semi-arid Mediterranean grassland, Jordan	Burning and fertilization increase vegetative cover by 35.5%, biomass increases production by 90.8%, increases species richness by 69.8% and reduces litter cover by 13.9%.	(Alhamad et al. 2012)
Mowing/ grazing frequency	Dry condition	Temperate grassland, France	Mowing increases grass height under dry condition.	(Dusseux et al. 2015)
Mowing frequency and N fertilization	Spring warming, Summer drought	Temperate grassland, Germany	ANPP increases by one time mowing + fertilization compared to 2-3 times mowing + fertilization under climate treatments.	(Bütof et al. 2012)
Mowing (yes or no)	Drought	Alpine grassland, Germany and Austria	Soil N status increases by mowing under drought.	(Fuchslueger et al. 2014)
Mowing frequency	Winter warming, winter rain, precipitation variability including drought	Temperate grassland, Germany	More frequent cutting strongly stimulates decomposition under warming, this stimulating effect is absent under extreme rainfall variability including drought. The stimulation of decomposition under more frequent cutting is attributed to changes in litter quality, namely a decrease in C/N ratio.	(Walter et al. 2013)
Mowing frequency, plant facilitation	Drought	Mesic and xeric calcareous grassland, France	Mowing under drought increases plant diversity by decreasing competition in mesic grassland, and decreases plant diversity in Xeric grassland. Plant facilitation cannot buffer the negative effects of drought in xeric grassland.	(Maalouf et al. 2012)
Mowing frequency	Drought	Temperate grassland, Germany	Frequent mowing reduces the resistance of grasslands against drought and increases species richness in one of the two study years. Low mowing frequency is more important for high resistance of grasslands against drought than species richness.	(Vogel et al. 2012)
Mowing frequency	Precipitation variability	Temperate grassland, Germany	Mowing frequency decreases shoot–root ratio, increases leaf N status. Mowing frequency increases productivity in the first year, but decreases at the end of second year. Mowing frequency has no overall effect on productivity. More frequent mowing does not buffer the adverse effects of rainfall variability on productivity, but makes leaf N concentrations more responsive to altered rainfall patterns in peak growing season.	(Walter et al. 2012)
Mowing frequency	Dry and wet condition	Grassland, Slovakia	Mowing increases species diversity. Wet grasslands are much more sensitive to mowing regime. Dry grassland react slowly.	(Galvánek and Lepš 2009)
Grazing intensity	Drought	Semiarid grassland, Arizona	Grazing increases plant invasion and decreases native plant diversity. Diversity of native plant is increased by intermediate grazing compared to high and no grazing. Grazing also reduces plant cover under drought.	(Loeser et al. 2007)

Management options	Climate treatments	Ecosystem, location	Key findings	Source
Legume facilitation	Drought, Heavy rainfall	Temperate experimental grassland, Germany	Legume facilitation increases plant community biomass and has minor effects on mycorrhization and increase stress resistance under climatic treatments.	(Walter et al. 2015)
Legume facilitation	Drought, Heavy rainfall	Temperate experimental grassland, Germany	Legume facilitation increases biomass production under heavy rainfall. Drought effects on biomass production of neighboring non-legumes can be minimized by legume facilitation. Facilitation effects on soil N status observed under control also persist under drought.	Manuscript 2
Species diversity	Drought	Grassland species, globally	Species richness can help maintain ecosystem functioning under drought.	(Craine et al. 2012)
Species composition	Drought and heavy rainfall	Experimental grassland, Germany	Community composition modifies the effects of drought and heavy rainfall on plant productivity and tissue die-back.	(Kreyling et al. 2008c)
Species diversity, N fertilization	CO_2	Grassland, Minnesota	Gross N mineralization increases with greater diversity and N addition, but not under elevated CO_2.	(West et al. 2006)
Species composition	Winter rain and spring rain addition	Grassland, California	Species interactions strongly influence plant responses to changing climate, overturning direct precipitation effects within 5 years.	(Suttle et al. 2007)
Within-species variation	Spring frost	European populations of grass species, Germany	Geographic origin and past climatic experience influence responses to late spring frost.	(Kreyling et al. 2012)
Within-species variation	Drought and warming	European populations of grasses species, Germany	European populations differ in biomass production and tissue die-back to drought and warming.	(Beierkuhnlein et al. 2011)
Rewetting after drought	Drought	Grassland California	Drying and rewetting cycles enhance subsurface soil microbial biomass, soil microbial activity and soil N mineralization.	(Xiang et al. 2008)
Rewetting after drought	Drought	Alpine grasslands, Austria	Rewetting after drought enhances soil N status at least briefly.	(Fuchslueger et al. 2014)
Grass hybrid	Flood	Grassland species *Festulolium* cultivar	Hybrid grasses increase fodder production under flood.	(Macleod et al. 2013)
Soil C addition/ mulching	Precipitation	Temperate grassland, China	Precipitation significantly increases the ratio of grass to forb biomass in C addition plots but shows only minor effects under ambient conditions. No precipitation effect on soil microbial activity is observed under C addition.	(Ma et al. 2012)

1.5 Artifacts in precipitation manipulation experiments

Precipitation manipulation experiments are useful tools for understanding the causal mechanism of ecological process under extreme drought. I have conducted a systematic literature survey (i) to identify whether there are any micro-climatological artifacts created by rain-out shelters and (ii) to identify the effects of these micro-climatological artifacts on plant responses in existing precipitation manipulation experiments. A total of 38 studies were identified that measured microclimate parameters in shrubland/heathland (n=20), grassland (n=15), forest (n=2) and other (n=4) ecosystems during precipitation manipulation by rain-out shelters (Figure 3). Among the 38 studies, most of them reported temperature (n=37) followed by photosynthetically active radiation (n=7) and relative humidity (n=4) inside and outside rain-out shelters (Figure 3).

Microclimate artifacts in precipitation manipulation experiments were further investigated for grassland studies (n=15) (see table 3). Fixed rain-out shelters create confounding effects on radiation and temperature in climate change manipulation experiments (Fay et al. 2000; English et al. 2005). Air temperature increases by 0.5°C to 2.2°C during pulsed drought manipulation experiments (English et al. 2005; Kreyling et al. 2008c). Daytime maximum and nighttime minimum soil temperature increases by 1.2 to 1.8°C under shelters (Fay et al. 2000). However, rain-out shelters reduce mean temperature by 1.8°C in precipitation addition and chronic drought manipulation experiments (Heisler-White et al. 2008). Photosynthetically active radiation (PAR), net radiation, photosynthetic photon flux density are reduced (10 to 28 %) in both pulse and chronic reduction experiments (Fay et al. 2000; Yahdjian and Sala 2002; Shinoda et al. 2010; Vogel et al. 2012). No significant effects on wind velocity, relative humidity and soil moisture were detected by English et al. (2005) in a chronic drought manipulation. Studies on the effects of rainout shelter artifacts on plant responses are scarce (see section 1.5, Table 3). Few studies observed considerable effects of shelter artifacts on biomass production and flowering phenology (Table 3).

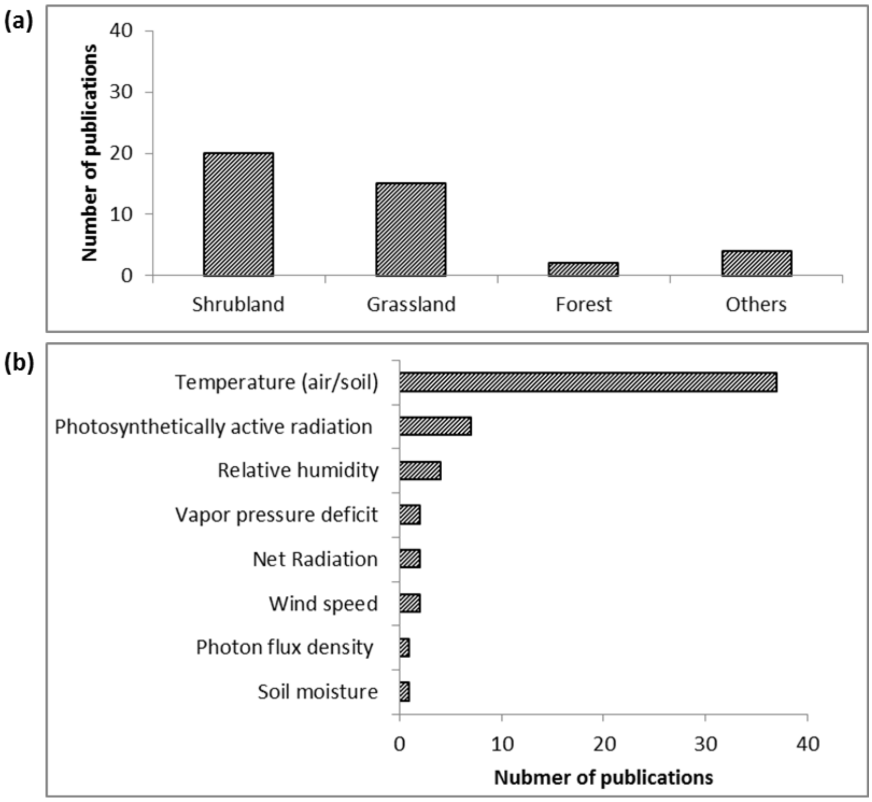

Figure 3: Research on rainout shelter artifacts in precipitation manipulation experiments based on publications found in the ISI Web of Science (a) number of publications in different ecosystems,

(b) number of publications which mentioned shelter artifacts on different microclimate parameters. Search criteria: ISI Web of Science search in February 2014 with the search string (drought OR climate* extreme) AND "field experiment*" AND Timespan: 2000 – 2014 and the additional condition that the climate manipulation was carried out using rain-out shelters, microclimate artifacts were mentioned and that multi-species systems were studied (as opposed to agricultural monocultures) in the field (not in pots). Based on these criteria, 38 studies were selected from 1069.

In summary, precipitation manipulations are generally carried out in field experiments either as pulsed full exclusion for a short period (pulsed drought) or partial exclusion for a long period (chronic drought) manipulations. In pulsed drought manipulations investigated areas are covered completely with rain-out shelters. Generally, in partial/chronic drought manipulations, the investigated areas are partly covered with translucent stripes that take away a defined percentage from any ambient rainfall event (Yahdjian and Sala 2002) for prolonged time periods. Results indicate that chronic drought manipulations with translucent stripes are less sensitive to treatment artifacts than pulsed drought manipulations with fixed shelters, yet both techniques share the major challenge of trying to create drought when ambient conditions are humid.

Table 3: Rain-out shelter artifacts on microclimates and plant responses in existing precipitation manipulation experiments in grassland ecosystems (for search details see figure 3).

Climate manipulations	**Rain-out shelter and drought type**	**Microclimate artifacts under shelter**	**Microclimate artifacts vs. plants responses**	**Source**
Drought (30% reduction), Rainfall variability (lengthen the dry intervals by 50%)	Fixed, partial exclusion, chronic drought	No effects on day or nighttime air temperature or midday vapor pressure deficit (VPD). Daytime maximum and nighttime minimum soil temperatures increase by 1.2–1.8°C. Photosynthetic photon flux density and net radiation reduces by 22%. Nighttime net radiation loss reduces by 65%. No artifacts on soil moisture.	Shelter effects reduce soil CO_2 flux by 15%. No shelter effects on ANPP and flowering phenology.	(Fay et al. 2000; Fay et al. 2002; Travers et al. 2010)
Drought (30%, 55% and 80% reduction)	Fixed, partial exclusion, chronic drought	Maximum midday air temperature reduces by 3.4°C, soil temperature decreases by 2-6.5°C, radiation decreases up to 10%.	NA	(Yahdjian and Sala 2002)
Drought (50% reduction), Precipitation (50% increase)	Fixed, partial exclusion, chronic drought	Minimum-daily temperature increases by 2°C, relative humidity remains within 4% of ambient conditions, photosynthetically active radiation (PAR) reduces by 24%. No considerable effects on wind velocity.	NA	(English et al. 2005)
Summer drought	Fixed, full exclusion, pulse drought	Rain-out shelters do not affect mean daily air temperature.	NA	(Kahmen et al. 2005)

Climate manipulations	**Rain-out shelter and drought type**	**Microclimate artifacts under shelter**	**Microclimate artifacts vs. plants responses**	**Source**
Increased precipitation amount and variability (190 mm in 120 days)	Fixed, full exclusion, pulse drought	Mean daily and maximum daily temperature reduces by 1.8°C and 3.4°C respectively.	NA	(Heisler-White et al. 2008)
Drought (32 days), Heavy rainfall (152 mm in 14 days)	Fixed, full exclusion, pulse drought	Near-surface air temperature slightly increases by 1.3°C during the weather manipulation period	No artifacts on biomass and flowering phenology	(Kreyling et al. 2008a,b,c; Jentsch et al. 2011; Walter et al. 2012, 2013)
Drought (72 days)	Fixed, full exclusion, pulse drought	Air temperature increases by (0.5–2°C) and ground-surface temperature by (2.5–4 °C), PAR reduces by 22%.	NA	(Shinoda et al. 2010)
Drought (42 days)	Fixed, full exclusion, pulse drought	Air temperature increases by 1.5–2.2°C, PAR reduces by 28%.	No artifacts on aboveground biomass production	(Vogel et al. 2012)

2 Objectives, motivation, summarizing results and synthesis

2.1 Objectives and motivation of the thesis

Objectives

Three main objectives of the thesis were (i) to investigate climate change (e.g. seasonal warming, modified precipitation amount and variability, and drought etc.) impacts on responses of temperate grassland plants and major ecosystem functions, (ii) to examine effects of three potential land management options that can help maintain ecosystem functioning of temperate grasslands in a changing climate, and (iii) to inspect whether there were any artifacts in ecosystem drought manipulation experiments created by rainout shelters. Motivation, major findings and synopsis of this thesis are presented in the section below:

Motivation

Grasslands cover around 30% of the Earth's terrestrial surface and provide many ecosystem services (Thébault et al. 2014). Climate change poses challenges to these services and grassland ecosystem functioning (Jaeschke et al. 2014). Therefore, grasslands have become an important study object in experimental biodiversity and climate change impact studies. North-American tall grasslands or natural prairies are well represented in biodiversity and climate change research. However, managed temperate grasslands (e.g. hay meadows/pastures) cannot be directly compared to continental natural prairies because of the differences in plant composition, climate, length of growing season, and importantly because of the considerably different management activities such as mowing that synchronized the extraction of above ground biomass production. Additionally, temperate grasslands such as meadows and pastures are of large spatial extent and high economic value not only in Europe but also in many temperate and oceanic regions of the world. Therefore, this thesis focused mainly on plants and ecosystem functioning of managed temperate grasslands.

Climate change consequences such as severe drought, heavy rainfall, seasonal warming are likely to have strong effects on vegetation and ecosystem functions (see section 1.3). The great majority of the existing studies investigated the effects of climate change on productivity. However, studies quantifying how climate change (such as 1000-year drought, high precipitation variability, seasonal warming, late frost in spring etc.) affects flowering phenology, plant eco-physiology, community composition, legume facilitation, plant nitrogen (N) and soil N status in managed temperate grasslands are lacking.

Although studies on the impacts of climate change on plants and ecosystem functions are increasing in number (see section 1.3), the interaction effects of seasonal climate and land management on grassland ecosystems are largely overlooked. Land management practices can minimize climate change impacts on grassland ecosystems. However, effective management strategies to mitigate the negative effects of climate change on plants and ecosystem functions in managed temperate grasslands have not been developed to date.

- ✓ Presence of legume species has positive effects on grassland productivity. Such key species are vital to maintain ecosystem productivity and stability. The effects of declining species richness on ecosystem functioning can partly be buffered by the presence of legumes. Therefore, ensuring the presence of legumes can be a potential management option in grasslands. However, there is a substantial lack of experimental research regarding effects of legume facilitation on productivity of neighboring grassland species under extreme climatic conditions.
- ✓ Species diversity increases the resistance of ecosystem productivity to climatic extremes because of differences among species in climate adaptations and disturbance tolerance. Therefore, increasing species diversity is one of the potential management strategies to increase productivity of grassland ecosystems under extreme climatic conditions. However, within-species genetic and phenotypic variation may be as high as among-species variation; potentially can buffer negative effects of climate change on ecosystem productivity. It is found that within-species biomass production differ under extreme climatic events. Yet, within-species differences in response to environmental fluctuations are rarely studied; in particular studies on (a) examining within-species plant N status variation after drought combined with rewetting and (b) investigating within-species seedling emergence variation under different climate conditions, are not available. In addition, the relative magnitude of within-species vs. among-species variation has remained largely unexplored. If within-species variation is as high as among species variation, then increasing within-species diversity can be recommended as a potential management strategy to increase ecosystem productivity as well as resistant/resilience of grassland ecosystems to climatic extremes.
- ✓ Furthermore, drought can decreases plant N status in grassland ecosystems by reducing soil N availability and plant N uptake. It is known that drought combined with rewetting can increase soil N availability. Yet, it is unclear to which degree grassland plants benefit from additional soil N availability if rewetting and harvest delays are applied in the context of drought. If rewetting and harvest delays buffer the negative effects of drought on plant N status and biomass production, then this can be recommended as one of the potential management strategies of grasslands under drought.

All the precipitation manipulation experiments of this thesis were carried out using rain-out shelters. The rain-out shelter technique is widely used to study ecological responses to climatic extremes (see section 1.5, table 3). The utilization of rain-out shelters is often criticized for creating micro-climatological artifacts, which can alter plant responses. Yet, a detailed quantification of micro-climatological artifacts created by rain-out shelters and how these artifacts change plant physiological activities are largely missing in existing climate change manipulation studies. Therefore, the rain-out shelter artifacts with respect to temperature, radiation, and soil moisture and their effects on plant responses (stomatal conductance, effective quantum yield of photosystem II, leaf water potential, plant cover and biomass) were investigated.

2.2 Climate change impacts on plants and ecosystem functions

2.2.1 Winter warming advances flowering phenology stronger than summer warming

Manuscript 1 showed that winter warming advanced average onset of flowering of a species-rich temperate grassland community by 3.1 days while summer warming advanced flowering by 1.5 days. In addition, winter warming significantly advanced the average onset of 22 early flowering species by 4.9 days, while summer warming advanced average onset of flowering by 2.3 days. However, neither winter nor summer warming affected the average onset of flowering of 23 late flowering species. These phenological responses are in accordance with the previous findings indicated that warming advances the flowering phenology of grassland species (Fitter and Fitter 2002; Cleland et al. 2006; Bloor et al. 2010). Memmott and others (2007) indicated that global warming advances the onset of flowering of plants by 2–6 days per 1°C increase in temperate regions. However, **manuscript 1** additionally showed that phenological responses were different dependent on the season when the warming took place. Flowering phenology of the studied temperate grassland community was more sensitive to a 1°C temperature increase in winter than to a 1°C increase in summer. **Manuscript 1** also suggested that early flowering species are more sensitive to seasonal warming, mainly to winter warming, while late flowering species appeared generally less sensitive. The observed advanced phenological shift of early flowering species likewise agrees with the previous findings (Fitter and Fitter 2002; Sherry et al. 2007).

Overall, **manuscript 1** shows that seasonal differences in warming, and particularly winter warming, alter community dynamics among early and late flowering species, which can cause shifts in the seasonal performance of many ecosystem processes in the studied semi-natural temperate grassland ecosystem. The observed semi-natural plant community and its species are representative for European managed grasslands. Therefore, the detected community level phenological responses are also representative for managed temperate grassland ecosystems across Europe.

2.2.2 Seasonal warming alters plant cover and biomass of early and late flowering species

The warming treatments significantly altered plant cover and biomass production of early and late flowering species (**Manuscript 1**). Winter warming significantly increased the biomass production (66%) and tended to increase the cover (13%) of late flowering species compared to the control while early flowering species showed neither a significant change in cover nor in biomass in response to winter warming. Summer warming showed no significant change in biomass or cover for any of the two species groups compared to the control. It is likely that warming enhances biomass production in temperate grassland (Thébault et al. 2014). However, **manuscript 1** shows that warming effects on plant cover and biomass production differ between early vs. late flowering temperate grassland species.

2.2.3 Modified precipitation amount and variability do not affect community level phenological shifts

The average onset of flowering of a species-rich grassland community was not affected by modified precipitation amount and variability during the growing season (**manuscript 1**). This is potentially because responses of a species-rich (45 species) community was considered, indicating that phenology of a species-rich grassland remain stable under precipitation extremes *sensu* the insurance hypothesis (Yachi and Loreau 1999). However, at the species level, onset of three species shifted earlier and onset of one species delayed under precipitation treatments. Such species-specific phenological responses are in accordance with previous findings (e.g. Jentsch *et al.* 2009; Nagy *et al.* 2013). The observed species-specific phenological shifts in **manuscript 1** indicate that intra-annual precipitation variability have the potential to alter species dynamics and functioning of the investigated temperate grassland ecosystem.

2.2.4 Drought reduces plant physiological activities, productivity and delays maturation

Drought decreased plant N status (leaf N concentration by 16%, shoot N uptake by 23%) and reduced productivity by 12% in an experimental temperate grassland (**manuscript 2**). In addition, 13% fewer individuals of four grass species reached the phenological stage “start of inflorescence elongation” under drought compared to control (**manuscript 3**), indicating drought can delayed plant phenological development (Buxton 1996; Grindlay 1997). Furthermore, **manuscript 6** showed that drought reduced plant stomatal conductance, effective quantum yield of photosystem II, and leaf water potential of a grassland plant (*Plantago lanceolata*). Taken together, although strong resistance to drought of an experimental grassland productivity was reported by Jentsch et al. (2011), plant physiological activities and phenological development are greatly affected by drought.

2.2.5 Climatic conditions lead to provenance/ecotype/population specific performances

The performance of plant species in new environments often depends on adaptations of local populations (Parker et al. 2003; Macel et al. 2007; Giménez-Benavides et al. 2007; Kim and Donohue 2013). Provenance-specific biomass production of grasses have been reported from drought and warming manipulation experiments (Beierkuhnlein et al. 2011). In addition, **manuscript 3** showed that drought combined with rewetting led to provenance-specific changes in plant N status. Although **manuscript 3** found differences between European populations of major grass species in plant N under drought and rewetting, there was no geographic pattern that could be correlated with climate variability at the location of origin. These results can be explained by the insufficient geographical coverage of populations or due to the unavailability of appropriate climatic parameters that represent the likelihood of recurrent drought and rewetting events at the location of origin.

Furthermore, **manuscript 4** emphasized that germination and seedling emergences of native and non-natives populations of a global invader (*Verbascum thapsus)* were differed by experimental climate conditions (temperature and moisture gradient). Native populations stemming from cool seed regions had a higher germination in cool experimental climate and a lower germination in warmer experimental-climates compared to non-native populations and vice versa. Previous studies suggested that non-native populations have higher germination responses than native ones (Beckmann et al., 2011; Hierro et al., 2009; Hirsch et al., 2012). However, **manuscript 4** shows that germination and seedling emergences of non-native populations are not always superior to the native populations. It depends on the climatic conditions under which the plant populations are grown and on the extent of local adaptation of a specific population to climate at their respective seed region.

2.3 Three potential land management options in a changing climate

2.3.1 Legume facilitations under climatic extremes: a way to maintain plant productivity

Manuscript 2 showed that legume presence facilitated community productivity of neighboring non-legumes under heavy rainfall. The presence of a legume could not effectively buffer the negative effects of drought with respect to productivity at the community level. However, species-specific legume facilitation effects were also detected in **manuscript 2**. *Arrhenatherum elatius* was facilitated both under drought and heavy rainfall, *Plantago lanceolata* was facilitated only under heavy rainfall, and *Holcus lanatus* was facilitated only under ambient conditions. These findings emphasize the importance of legume presence and species-specific traits for maintaining ecosystem productivity in the face of extreme climatic events. The positive effects of legume presence found under ambient conditions also persisted under drought for plant N nutrition and soil N status.

In addition, unaffected plant N status of the legume species itself indicated that N_2 fixation by the legumes appeared not to be impaired by the drought. Besides, **manuscript 2** showed slightly higher soil N-concentrations in the drought plots. It could not be detected whether N_2 fixation had taken place during the peak of the drought treatment as drought was applied as pulsed stress events. N_2 fixation could have occurred before and after the drought treatment. Therefore, **manuscript 2** suggested that effects of legume facilitation are slightly reduced under low soil moisture conditions as a consequence of reduced N-uptake rather than N_2 fixation.

2.3.2 Increasing within-species diversity in a changing climate: an adaptation strategy

Manuscripts 3 and 4 showed that within-species populations differed in plant N status under drought and also in seedling emergence rate under representing climates of seven biomes, indicating that different populations have distinctive stress tolerance ability. In addition, **manuscript 5** found variation in within-species responses are as high as variation in among-species responses under drought, spring frost, and winter warming plus frost. These results have important implication for biodiversity conservation, maintaining ecosystem functions as well as ecosystem management in the context of climate change.

Species diversity insures ecosystem functioning in the face of climate change (Yachi and Loreau 1999; Isbell et al. 2015). In a species-rich system, plants which are less important for ecosystem functioning might perform better, become dominant and start to act as key drivers of ecosystem processes in a changing climate (Walker et al. 1999). Species are considered as an important component of an ecosystem because they are genetically different and have specific disturbance tolerance capability. Manuscripts 3, 4 and 5 show that within-species populations also have different disturbance tolerance capabilities (here climate change). Therefore, increasing within-species diversity may help maintain ecosystem productivity in the face of climate change.

Genetic differences have been found within *A. elatius* populations used in the **manuscript 3** (Michalski et al. 2010). Yet, the relation between genetic differences in populations and differences in performance was not investigated in **manuscript 3, 4 and 5**. The findings imply that diversity within species may be as important in increasing resistance of ecosystem productivity and stability in the face of climatic extremes as species diversity. Assisted migration of within-species population may increase the stability of an ecosystem (Kreyling et al. 2011). However, before doing so, negative and positive effects of assisted migration of non-native populations on functioning of native population should be investigated.

2.3.3 Rewetting and delaying harvest after drought: increases in plant nutrition

It is unclear to which degree plants benefit from additional soil N availability after a drought combined with rewetting event. In **manuscript 3,** an experimental drought stress was manipulated and the biomass was harvested after 10 days of rewetting event, where biomass was reduced by 29% compared to control, but plant N status improved significantly (N concentration increased 96%; N content increased 31%, and C/N ratio decreased 46%).

Higher plant N status after drought combined with rewetting and 10 days harvest delay can be explained by higher N uptake, as was indicated by higher shoot N content. Higher plant N status might also be due to indirect effect of rewetting on soil N availability. It is found that rewetting of dry soils can increases soil N availability (Birch 1964; Borken and Matzner 2009) through higher soil N mineralization (Bloem et al. 1992). Plant N uptake and plant N status linearly increases with soil N availability (Yuan et al. 2007; Lü et al. 2014). Therefore, if a drought event is peaking during the growing season, a potential management strategy would be harvesting grassland biomass at least 10 days after a subsequent rain event. Yet, results suggest that rewetting and harvest delay could not compensate the negative effects of drought on productivity, but could improve plant N status **(Manuscript 3)**.

2.4 Rainout shelter artifacts in precipitation manipulation experiments

2.4.1 Ambient weather conditions influence drought effects under rainout shelters

Negative drought effects on short term ecophysiological responses of plant were strongly correlated with the ambient microclimates conditions (**manuscript 6**). Air temperature and vapor pressure deficit (VPD) were strongly correlated with ecophysiological response of plants. Therefore, **manuscript 6** suggests that the effectiveness of drought simulations by rain-out shelters depends strongly on the ambient outside weather conditions. Drought stress is only occurs during warm and dry outside conditions. This high dependency of experimental drought effects on ambient microclimates suggest that results from different climate manipulation experiments and even from different years within the same experiment are hardly comparable, as they alter by ambient weather conditions. In general, this finding is important for the evaluation, generalization, and up-scaling of the increasing amount of research findings available from ongoing precipitation manipulation experiments.

2.4.2 Rainout shelter artifacts have no significant effects on vegetation responses

Rainout shelters reduced 22% and 18% of the midday incoming shortwave radiation and net radiation (Wm^{-2}), respectively (**Manuscript 6**). Under the rainout shelters day time mean air temperature at 5cm and mean soil temperature at -2cm were decreased by 1.1°C and 0.9°C, respectively. On the other hand, rainout shelters slightly increased the night time air and soil temperature by 0.4°C and 0.3°C, respectively. Averaged over four consecutive years, soil moisture was only 0.13% lower in the rainout shelter artifact control plots. The micro-climatological artifacts under rain-out shelter are in the direction of natural drought events, which are commonly accompanied by increased air temperatures (De Boeck et al. 2010). However, the elimination of late frost events by slightly increasing night time temperature might create a hidden treatment (Huston 1997).

Manuscript 6 showed that fixed rain-out shelters used for short-term manipulations of pulsed drought events create no significant impact on the plant ecophysiological responses than previously thought. Furthermore, long term community productivity and aboveground vegetation cover did not differ significantly by rainout shelter artifacts. For pulsed precipitation manipulation, this finding implies that fixed rain-out shelters can be used without creating strong artifacts on plant responses. Fixed shelters thus remain a useful tool for ecological climate manipulation research (Beier et al. 2012).

2.4.3 How to make precipitation manipulation experiments comparable?

The strong correlation between the realized ecophysiological drought effects and the ambient microclimate shown in **manuscript 6** offers options for generalizations: drought effects can be standardized by monitoring ambient microclimate conditions and should be adjusted to be comparable across space and time. Microclimatic parameters such as daytime daily mean of air temperature and VPD can explain much of the inter-annual variation in realized drought effects on plant ecophysiological activities in a pulsed drought field experiment **(Manuscript 6)**. Therefore, temperature and VPD should be quantified in all drought manipulation experiments and presented in any manuscript. So far, many studies report air temperature during drought manipulations, while VPD is rarely mentioned (Table 3 and **manuscript 6**). VPD has been shown to consistently correlate with plant productivity across ecosystems (Kiniry et al. 1998; Leuschner 2002; Brando et al. 2010). Therefore, meta-analyses will profit from reporting of ambient microclimate conditions such as temperature and VPD as they require more complex and realistic scenarios to explain response variation than simple annual reduction of precipitation (Wu et al. 2011; Kreyling and Beier 2013).

3 Emerging research challenges and frontiers under a changing climate

3.1 Plant responses to a warmer world in managed temperate grassland ecosystems

(i) Most temperate plants from dry sites respond negatively to warming while plants from higher altitude/wet/humid sites respond positively. But how close are they (plants benefitting from warmer temperatures) to their tipping point? To answer this question, the temperature thresholds of grassland plants need to be quantified. Climate chamber experiments examining plants growth performance under simulated temperature and moisture gradients could help in this case.

(ii) Why does the weather two months prior to flowering play a dominant role in shifting flowering phenology? Which phenological stages are more sensitive to seasonal climate warming? Manuscript 2 investigated the first flowering date (onset) of a grassland community under seasonal warming and precipitation changes. However, it may be the case that other phenological stages (e.g. peak flowering date and last flowering date) of some species are more sensitive to seasonal temperature and precipitation variation compared to the flowering onset. Therefore, complete assessments of flowering phenology distribution (such as onset, peak, last date and length of flowering) are important to get more insights about the seasonal changes in grassland ecosystem functions.

3.2 Importance of plant functional traits under extreme drought

(iii) Which plant traits can make an ecosystem resistant/resilient against drought? Species-specific responses are often reported under extreme drought events. Some species are resistant, some resilient and some are sensitive to drought. However, traits responsible for differential drought responses are not well investigated. Identifying key plant traits driving differential drought responses and examining their role in drought tolerance is essential for maximizing grassland services under drought.

(iv) What is the tipping point of grassland ecosystems under drought? Longer dry periods within the growing season are predicted for temperate grassland ecosystems. Yet, knowledge on threshold or tipping points of grassland ecosystems under drought is still missing. This information is indispensable to set proper management strategies against increasing drought periods.

3.3 Importance of within-species variation under extreme climatic events

(v) Can within-species diversity provide insurance against climatic extremes in grassland ecosystem? It is evident that ecotypes (provenances or populations) of grassland species react differently under climatic extremes. Genetic differences, local adaptation or past climatic experiences are often put forward to explain within-species variation. However, there is a substantial lack of experimental evidence regarding how long genetic variation (if it is the product of strong climatic adaptation) or local adaptation effects exist if ecotypes are planted in the same environment. This information is necessary to determine whether within-species diversity can effectively buffer against declining species richness or provide insurance against changing grasslands ecosystem functions in a changing climate.

3.4 Timing and interaction of extreme climatic events

(vi) Is the timing of extreme climatic events (occurs in spring/summer/autumn) more crucial than changes in magnitude (e.g. 100-year or 1000-year extreme) to affect ecosystem functioning? The great majority of the existing climate manipulation experiments have focused on how changes in magnitude of extreme climatic events affect plant responses. However, how the timing/seasonality of these events affects plant response is missing.

(vii) How does winter climate change in combination with summer drought alter ecosystem responses? In recent years, winter climate change (such as winter rain, frost, changes in snow cover etc.) has been most pronounced in temperate regions. Though winter is a non-growing season in temperate grasslands, climate change in this period has the potential to change vegetation dynamics indirectly via their effects on soil microbial communities, soil physical properties and nutrient availability. If such winter climate change is followed by drought in the growing season, the combined effects might be severe. Therefore, it is necessary to investigate interaction effects of extreme climatic events on temperate grassland plants.

3.5 Interaction between land management and extreme climatic events

(viii) Land management can minimize direct effects of climate change on plants and ecosystem functioning. Three potential management options such as legume presence, within-species diversity and rewetting after drought were tested under experimental conditions here. To apply the findings, studied management options need further investigation in natural ecosystems. Furthermore, frequent mowing is one of the common management practices in temperate grassland ecosystems. Yet, the influence of different mowing height on this system under extreme climatic events is not clear. In particular, mowing height can reduces the resistance of a grassland ecosystem to drought potentially because low canopy height increases wind speed at the ground level which can further increases soil surface evaporation and cause higher soil moisture loss. Therefore, if a drought event hits a system right after mowing (e.g. mown at 3cm height), the effects of drought on productivity could be severe.

4 Declaration of own contribution

Writing: Writing the manuscripts, including literature research.

Field work: Organization and execution of field data collection together with the help of technicians, students and interns.

Data analysis: Sorting and statistical analysis of data sets used in each manuscript.

Visuals: Ideas and creation of tables and figures used in the manuscript.

Concept and discussion: Ideas and structure of the manuscript, and justification of results.

Editing: Proof-reading, integrating feedbacks and inputs from co-authors**,** preparation for submission and resubmission to journal.

List of manuscript presented in this thesis and declaration of own contribution

Manuscript 1:

Title: **Phenological sensitivity to seasonal warming and altered precipitation differs between early and late flowering species in a semi-natural temperate grassland**

Authors: **Mohammed Abu Sayed Arfin Khan***, Carl Beierkuhnlein, Juergen Kreyling, Sabrina Backhaus, Sebastian Varga, Anke Jentsch

*Corresponding author

Journal and status: Journal of Ecology (submitted in November 2015)

Own contribution: Writing: 85%; Data analysis: 90%; Visuals: 100%; Concept and discussion: 50%; Editing: 70%.

Manuscript 2:

Title: **Climatic extremes lead to species-specific legume facilitation in an experimental temperate grassland**

Authors: **Mohammed Abu Sayed Arfin Khan***, Kerstin Grant, Carl Beierkuhnlein, Juergen Kreyling, Anke Jentsch.

*Corresponding author

Journal and status: Plant Soil (2014) 379:161–175

Own contribution: Writing: 80%; Data analysis: 90%; Visuals: 100%; Concept and discussion: 70%; Editing: 70%.

Manuscript 3:

Title: **Provenance-specific improvement of nitrogen status in grasses after drought combined with rewetting**

Authors: **Mohammed Abu Sayed Arfin Khan***, Juergen Kreyling, Carl Beierkuhnlein, Anke Jentsch

*Corresponding author

Journal and status: PLOS ONE (submitted in September 2015)

Own contribution: Writing: 90%; Data analysis: 100%; Visuals: 100%; Concept and discussion: 70%; Editing: 80%.

Manuscript 4:

Title: Factors influencing seedling emergence success of three global invaders under representative climates of seven biomes

Authors: **Mohammed Abu Sayed Arfin Khan***, Anke Jentsch

*Corresponding author

Journal and status: Perspectives in Plant Ecology, Evolution and Systematics (In preparation to submit in Mid November 2015)

Own contribution: Writing: 95%; Field work: 90%; Data analysis: 100%; Visuals: 100%; Concept and discussion: 90%; Editing: 90%.

Manuscript 5:

Title: Plant responses to climatic extremes: within-species variation equals among-species variation

Authors: Andrey V. Malyshev, **Mohammed Abu Sayed Arfin Khan**, Carl Beierkuhnlein, Manuel J. Steinbauer, Hugh A. L. Henry, Anke Jentsch, Jürgen Dengler, Evelin Willner, Juergen Kreyling

Journal and status: Global Change Biology, DOI: 10.1111/gcb.13114 (**accepted on 1st October, 2015, in press**)

Own contribution: Writing: 10%; Data analysis: 50%; Visuals: 50%; Concept and discussion: 10%; Editing: 10%.

Manuscript 6:

Title: **Drought effects in climate change manipulation experiments influenced by ambient weather conditions – effect sizes and rain-out shelter artifacts**

Authors: Juergen Kreyling*, **Mohammed Abu Sayed Arfin Khan***, Fahmida Sultana, Wolfgang Babel, Carl Beierkuhnlein, Thomas Foken, Julia Walter, Anke Jentsch

*authors contributed equally to the manuscript

Journal and status: Ecosystems (submitted in August 2015)

Own contribution: Writing: 50% (first draft); Field work: 30%; Data analysis: 80%; Visuals: 80%; Concept and discussion: 30%; Editing: 30%.

In the following I present the highlights of each manuscripts used in this thesis (Table 4). Furthermore, table 5 gives an overview of the oral or poster presentation of the findings of this thesis at national and international scientific conferences/workshops/meetings.

Table 4. Key findings of each manuscript presented in the thesis.

Theme	**Highlights**
Manuscript 1: Shift in flowering phenology of temperate grassland ecosystem under seasonal climate change	✓ Winter warming advances phenology of early flowering temperate grassland species more than summer warming. ✓ Late flowering species were generally less sensitive to both summer and winter warming. ✓ No shift in community level flowering phenology is detected under precipitation change. ✓ Phonological shifts are diverse and species-specific under seasonal climate change.
Manuscript 2: Effects of legume presence on ecosystem functions under climatic extremes in temperate grassland	✓ Legume presence facilitated community productivity of neighboring non-legumes under ambient weather and heavy rainfall conditions. ✓ Legume presence facilitated species-specific primary productivity and N nutrition of neighboring non-legumes under drought. ✓ Legume facilitation effects on soil N status observed under control also persisted under drought.
Manuscript 3: Responses of European grass populations under drought combined with rewetting	✓ Rewetting after drought improved the plant N nutrition of major grass species ✓ Within species physiological responses to climatic extremes were provenance-specific. ✓ Delayed harvest after drought is suggested as N status can be improved by rewetting. ✓ No general pattern observed between population responses and climate at the location of origin.

Manuscript 4: Seedling emergence of global invaders and their populations under experimental climates of seven biomes	✓ The warm and semi-arid to humid experimental-climates are suitable for crucial first steps of invasion success by *L. polyphyllus*, *S. inaequidens*, and *V. thapsus*. ✓ Phenotypic plasticity modifies responses of three species under different experimental-climates. ✓ Climate of seed region influences germination and seedling emergence of native and non-native populations of *V. thapsus*.
Manuscript 5: Within and among species variation under climatic extremes	✓ Within-species variation equals among-species variation under drought, frost and warming. ✓ Including both within and among species variability in ecological theory (e.g. insurance hypothesis) and for practical applications (e.g. biodiversity conservation) are recommended.
Manuscript 6: Rainout shelter artifacts and their effects on plants in climate change manipulation experiments	✓ Drought effects in precipitation manipulation experiments are influenced by ambient weather. ✓ Drought stress under rain-out shelters are correlated with ambient temperature and vapor pressure deficit. ✓ Shelter artifacts had no significant effects on plant responses.

Table 5. Presentation of research at scientific conferences/workshops

Date	**Conference/workshop**	**Title**	**Type**
Oct. 2012	BayCEER Workshop, Bayreuth, Germany	Role of legume presence in modifying plants response to extreme weather events in temperate grassland	Oral
Sep. 2013	GfÖ, 43rd Annual Meeting, Potsdam, Germany	Can legume presence modifies responses of temperate grassland species under annually recurrent pulsed drought and heavy rainfall events?	Oral
Sep. 2013	GfÖ, 43rd Annual Meeting, Potsdam, Germany	Plant responses in precipitation manipulation experiments are modified by outside weather conditions	Poster
Jul. 2014	BIOGEOMON, 8th International Symposium on Ecosystem Behaviour, Bayreuth, Germany	Outside weather conditions determine drought effects in climate change manipulation experiments – effect sizes, rain-out shelter artifacts, and ways forward	Poster
Jan. 2015	IBS conference 2015, Bayreuth, Germany	Seedling emergence of invasive plants is limited by low temperature and humidity in experimental target-climate and can be predicted by climate of native and non-native origin	Poster
Aug. 2015	ESA 100th Annual Meeting, Baltimore, Maryland, USA	Ambient conditions influence drought effects in climate change manipulation experiments – effect sizes, rain-out shelter artifacts, and ways forward	Oral
Sep. 2015	ClimMani annual meeting, Poznan, Poland	Provenance-specific improvement of nitrogen status in grasses after drought combined with rewetting: implication for grassland ecosystem management	Poster

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6 Manuscripts

6.1 Manuscript 1: Phenological sensitivity to seasonal warming and altered precipitation differs between early and late flowering species in a semi-natural temperate grassland

6.2 Manuscript 2: Climatic extremes lead to species-specific legume facilitation in an experimental temperate grassland

6.3 Manuscript 3: Provenance-specific improvement of nitrogen status in grasses after drought combined with rewetting

6.4 Manuscript 4: Factors influencing seedling emergence success of three global invaders under representative climates of seven biomes

6.5 Manuscript 5: Plant responses to climatic extremes: within-species variation equals among-species variation

6.6 Manuscript 6: Drought effects in climate change manipulation experiments influenced by ambient weather conditions – effect sizes and rain-out shelter artifacts

6.1 Manuscript 1

Title: Phenological sensitivity to seasonal warming and altered precipitation differs between early and late flowering species in a semi-natural temperate grassland

Journal: Journal of Ecology (submitted in November 2015)

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Abstract

- Shifts in flowering phenology of plants are indicators of climate change. The great majority of existing phenological studies refer solely to gradual warming. However, knowledge on how flowering phenology responds to changes in seasonal variation of warming and precipitation regimes is missing.
- We report the onset of 22 early (flowering before/within May) and 23 late (flowering after May) flowering species in response to manipulated seasonal warming (+1.2°C; last 100 years summer/winter warming), additional winter rainfall, and modified precipitation variability (including 1000 years extreme drought) over the growing season in two consecutive years for a species-rich temperate grassland.
- Average onset of flowering (over two years) of the grassland community was significantly advanced 3.1 days by winter warming and 1.5 days by summer warming compared to control. This phenological shift was mainly due to the higher sensitivity of early flowering species to winter warming. Average onset of early flowering species was significantly advanced, 4.9 days by winter warming and 2.3 days by summer warming respectively. Average onset of late flowering species was not significantly altered by any seasonal climate warming treatment. However, winter warming significantly increased the biomass of late flowering species, while winter warming had no effects on plant cover and biomass

of early flowering species compared to control. Average onset of flowering of early and late flowering community were neither affected by winter rain addition nor by growing season precipitation variability. However, a few species responded differently to winter rain addition, and modified growing season precipitation variability.

- *Synthesis*: Early flowering species were generally more sensitive to seasonal warming than late flowering species, irrespective of warming occurring over winter or over summer. These shifts in flowering phenology were independent from changes in species cover and biomass. Overall, winter warming proved more effective than summer warming. Winter rainfall addition and increased precipitation variability over the growing season showed no effects on flower phenology of this species-rich grassland. Our data suggest seasonal differences in warming, and particularly winter warming, might alter community dynamics among early and late flowering species which can cause shifts in seasonal performance of the studied ecosystem.

Introduction

The global surface temperature has increased by 0.85°C during the period 1880 to 2012 (IPCC, 2013). Besides further gradual warming, modifications in seasonality are expected (Hegerl *et al.*, 2011b; Min *et al.*, 2011; IPCC, 2013). More extreme weather regimes due to an increased climatic variability are also expected (Hegerl *et al.*, 2011a), especially in temperate environments (IPCC, 2012). In Central Europe, temperature is likely to increase more rapidly in winter than in summer (Kreyling & Henry, 2011; Andrade *et al.*, 2012; Vautard *et al.*, 2013, 2014). On the other hand, annual precipitation sums are expected to remain unaffected in this region, but the intra-annual precipitation variation may increase (Jacob, 2009; Kotlarski *et al.*, 2014). Increased precipitation variability is also causing occasional prolonged periods of moist or dry conditions (Francis & Vavrus, 2012; Petoukhov *et al.*, 2013). In Europe, precipitation is likely to decrease in summer and increase in winter (Zolina, 2012).

Flowering phenology is sensitive to climate changes (Parmesan & Yohe, 2003; Root *et al.*, 2003; Badeck *et al.*, 2004; Sherry *et al.*, 2007; Körner & Basler, 2010; Cook *et al.*, 2012; Ovaskainen *et al.*, 2013). Previous studies suggest that flowering phenology is generally shifting to earlier dates in temperate environments (Menzel & Fabian, 1999; Menzel *et al.*, 2006; Hovenden *et al.*, 2008; Bloor *et al.*, 2010; Cornelius *et al.*, 2014). The shift in timing of flowering phenology can change plant community composition (CaraDonna *et al.*, 2014) and plant-pollinator interactions (Memmott *et al.*, 2007; Scaven & Rafferty, 2013). Climate change driven phenological shifts also have an impact on the number of flowers, seed set, timing of seed ripening and seed dispersal (Santandreu & Lloret, 1999; Saavedra *et al.*, 2003), which in turn affect reproductive fitness of plants. Earlier flowering influences many other plant activities, like leaf expansion, root growth, and nutrient uptake (McKane *et al.*, 1990), which can play important roles in niche differentiation among coexisting species. Furthermore, species specific flowering phenology alters structure of plant communities, gene flow between species and contributes to plant invasion success as climate warms (Fitter & Fitter, 2002; Wolkovich *et al.*, 2013). However, studies implying shifts in phenology with shifts in ecosystem functioning are scarce (Jentsch *et al.*, 2011).

It is evident that the seasonal timing of climatic changes can be more critical for organisms and ecosystems than annual sums or uniform shifts. The great majority of the existing phenological studies investigated the flowering phenology only under year-round gradual warming. Yet, studies on how seasonal variation in warming (winter versus summer) shifts flowering phenology of plants are missing. Flowering phenology of temperate plants is responsive mainly to average monthly temperature of the preceding two months and the month of flowering onset (Chmielewski & Rötzer, 2001; Menzel *et al.*, 2006; Nagy *et al.*, 2013). Therefore, it is likely that winter warming (October – March) advances phenology of early flowering species (flowering before/within May) and summer warming (April – September) advances phenology of late flowering species (flowering after May). Yet, this assumption needs to be tested.

Besides temperature change, modified precipitation regimes can strongly influence phenological behavior of plants. But so far, few studies have investigated the effects of strong changes in moisture availability (e.g. drought or heavy rainfall) on flowering phenology (Jentsch *et al.*, 2009; Bloor *et al.*, 2010; Nagy *et al.*, 2013). This is also due to the fact that precipitation regimes are more complex and variable by nature and more difficult to manipulate (Beier *et al.*, 2012). Late spring drought (32 days complete reduction) was found to delay flowering phenology of an early flowering legume shrub, e.g. *Genista tinctoria*, but had no effect on another late flowering shrub species, *Calluna vulgaris* (Nagy *et al.*, 2013). No effect of summer drought (20% reduction of precipitation) on the onset of four late flowering grasses (*Agrostis capillaris, Festuca arundinacea, Festuca rubra*, *Poa pratensis*) and on one early flowering grass (*Trisetum flavescens*) were found by Bloor *et al.* (2010). However, Jentsch *et al.* (2009) showed that late spring drought (32 days complete reduction) generally advances the overall mean mid-flowering date and extends the mean flowering length of three early flowering (*Plantago lanceolate, Genista tinctoria, Vaccinium myrtillus*) and seven late flowering (*Arrhenatherum elatius, Holcus lanatus, Geranium pratense, Lotus corniculatus*, *Agrostis stolonifera, Calluna vulgaris, Deschampsia flexuos*) grassland and shrub species in Central Europe.

Similarly, effects of heavy rainfall on flowering phenology reported up to date are species-specific. For instance, heavy rain (170mm over 14 days) in summer advanced flowering phenology of a late flowering legume forb (*Lotus corniculatus*) and of an early flowering legume shrub (*Genista tinctoria*) (Jentsch *et al.*, 2009; Nagy *et al.*, 2013). But, heavy rainfall (170mm over 14 days) in summer had no effect on five late flowering grassland species and dwarf shrubs (Grass: *Arrhenatherum elatius*, *Holcus lanatus*; forb: *Plantago lanceolata*, *Geranium pratense*; shrub: *Calluna vulgaris*) (Jentsch *et al.*, 2009; Nagy *et al.*, 2013). In addition, a grassland study by Cleland *et al.* (2006) also showed that 50% increased precipitation over the growing season had no significant effect on the onset of flowering of five early flowering (*Bromus diandrus, Bromus hordeaceus, Erodium botrys, Geranium dissectum*, *Vicia sativa*) and four late flowering species (*Avena* spp., *Lolium multiflorum, Vulpia myuros, Crepis vesicaria*).

Taken together, the existing knowledge on how moisture availability alters flowering phenology is not consistent. This is probably because of the timing and amount (reduction/addition) of precipitation events realized, and the composition of plant communities investigated are not comparable in the existing studies. Most of the phenological studies done with a sudden change in moisture availability involved single or small sets of species and emphasized diverse (species-specific) responses (Cleland *et al.*, 2006; Jentsch *et al.*, 2009; Bloor *et al.*, 2010; Nagy *et al.*, 2013). It is well-known that biodiversity increases the resistance of ecosystem productivity to fluctuating environments. Yet, biodiversity effects on ecosystem phenology to a changing climate are not clear. Therefore, it is timely to test responses over a large set of species, in order to gain a general overview of how flowering phenology responds to changing precipitation variability at the scale of species-rich plant communities and ecosystems. Although conflicting species-specific responses are reported so far, we assume that phenological responses of a species-rich community will be stable to growing season precipitation variability *sensu* the insurance hypothesis (Yachi & Loreau, 1999).

Besides the direct impact of modified moisture regimes in the period before and during phenological responses, it is important to understand how previous periods are influencing phenological responses. Changes in winter precipitation are expected in Europe (Zolina, 2012) which could have an impact on water storage in deeper soil horizons and on plant performance throughout the following growing season. Shifting limits of growing seasons can be intensified by increased winter rain, leading to earlier flowering onset and an earlier start to the growing season. However, how winter rain influences flowering phenology at the community level has not yet been documented.

We investigated the onset of flowering in a species-rich community (22 early flowering and 23 late flowering species) under seasonal climate change manipulations such as winter warming, summer warming, winter rain addition, and altered precipitation variability in the growing season (low precipitation variability, medium precipitation variability and high precipitation variability) in a semi-natural temperate grassland over two consecutive years. First, we hypothesized that (1a) both, winter warming and summer warming, are advancing the onset of flowering in grassland communities, whereby (1b) the onset of early flowering species will be advanced by winter warming and the onset of late flowering species will be advanced by summer warming. Second, we assumed that (2) winter rain addition and growing season precipitation variability will have no effect on the onset of flowering of a species-rich early and late flowering grassland community.

Materials and Methods

Experimental site

This study is part of the EVENT II experiment (Jentsch & Beierkuhnlein, 2010) which was established in 2008 on a semi-natural temperate grassland at the Ecological Botanical Garden of the University of Bayreuth, Germany (49°55'19"N, 11°34'55"E, 365 m a.s.l.). The regional climate is characterized as temperate and moderately continental with a long-term mean annual temperature of 8.2 °C and a long-term mean annual precipitation of 724 mm (1971–2000) with precipitation peaks in December/January and June/July. Phenological data recording was carried out in 2010 and 2011.

The soil of the EVENT II experiment is classified as Gleysol (Glaser *et al.*, 2013) with a homogeneous, loamy Ap-horizon of about 30 cm depth (43% silt, 42% sand, and 15% clay) and a clayey Bg-horizon. The mean pH-value of the topsoil is 4.1 (1 M KCL). The experimental site is a semi-natural meadow, which has not been fertilized for more than 20 years and not ploughed for at least 25 years prior to the establishment of the experiment in 2008. This hay meadow is mown twice a year (end of July/September), because without mowing the species composition would change over time mainly due to encroachment of woody species. Without cutting, there would be almost no grasslands in Central Europe. The semi-natural temperate grassland is dominated by tall grasses such as *Alopecurus pratensis* L. (meadow foxtail) and *Arrhenatherum elatius* (L.) P. Beauv. ex J. Presl & C. Presl (tall oat-grass). Grasses represent 75-85% of the total plant biomass, whereas the percentage of forb species varies between 15-23%. Legumes represent less than 2% with regard to the plant biomass yield. A list of available plant species during the phenological observations in 2010/2011 is provided in the supporting information table S1.

Experimental design

The experiment was carried out in a partly crossed three-factorial design: (1) growing season precipitation variability (levels: “low”, “medium”, “$\text{high}_{\text{early}}$”, “$\text{high}_{\text{previous year late}}$”), (2) warming (levels: “winter warming”, “summer warming”, “control”), and (3) “winter rain” (levels: yes or no). Within each precipitation variability treatment, application of winter warming, summer warming, winter rain addition and control were blocked and randomly assigned. The size of each block was 6 m x 4 m and divided into plots of 1.5 m x 1.5 m (nested design). Each factorial climate treatment plot was replicated five times.

Precipitation variability treatment

The low precipitation variability plots received at least long-term (1971–2000) weekly average precipitation. If natural rainfall was less than the long-term average sum for the same week, the missing amount was irrigated using portable irrigation systems. Drop size and rainfall intensity resembled natural heavy rainfall events through application by Veejet 80100 nozzles, commonly used in erosion research. At 0.03 MPa water pressure, this system resulted in 2.8 mm water per minute. If weekly rainfall exceeded the long-term average sum, it was not subtracted for the next irrigation. The precipitation amount of the low precipitation variability treatment served as a reference amount and that was kept constant for all precipitation variability treatments, while the temporal precipitation pattern was altered during the growing season (April–September).

The medium precipitation variability plots received ambient precipitation, but were irrigated additionally four times (beginning of May, end of June, beginning of August and end of September) in order to keep total precipitation constant according to the reference amount. These additional irrigations were also implemented on the two high variability treatments, resulting in the same annual amount of precipitation for all treatments. The two extreme precipitation variability treatments, early high precipitation variability and previous year late high precipitation variability plots were exposed to a 1000-year drought event in spring and summer, respectively. The duration of this event was calculated by Gumbel statistics and based on the 40 years (1961–2000) time series of a local weather station. The manipulation was done by excluding natural rainfall for 42 days using rain-out shelters. These were constructed with a steel frame (Hochtunnel, E & R Stolte GmbH, Germany), and covered with a transparent plastic sheet (material: 0.2 mm polyethylene, SPR 5, Hermann Meyer KG, Germany). Rain-out shelters permitted nearly 90% penetration of photosynthetically active radiation.

Rain-out shelters were removed from the two extreme precipitation variability treatments after 42 days of drought manipulation. The excluded amount of rainfall was applied with the adjustment to the reference amount simultaneously at the end of the artificial drought period as one heavy rainfall event within three consecutive days. Additionally, we ran a rain-out shelter artifact control treatment to check for shelter artifacts. The rain-out shelter artifact control plots were irrigated below the shelters with the same precipitation amounts as the medium precipitation variability treatment (ambient precipitation) after every rainfall. No significant difference in onset of flowering was found between ambient control and rain-out shelter artifact control ($F = 0.1$; $p = 0.7341$).

Coefficient of variation CV (= standard deviation × daily mean - 1 × 100) for precipitation were calculated to test the strength of the precipitation variability treatments (Table S2). In addition, soil moisture in the main rooting zone (-2 to -7 cm) in each treatment plot per precipitation block (n=5 for each treatment combination) were logged every hour using frequency domain (FD)-sensors (ECH$_2$O, Decagon devices, Pullman, USA). The soil moisture data are based on the average of all five replicates for each treatment except when missing data occurred due to sensor or logger errors. For both years the growing season precipitation variability and soil moisture were altered resulting in lowest variability in low, intermediate variability in medium, and high variability in high$_{\text{early}}$ (spring drought) and high$_{\text{previous year late}}$ (summer drought) precipitation variability treatments (Fig. 1, Table S2).

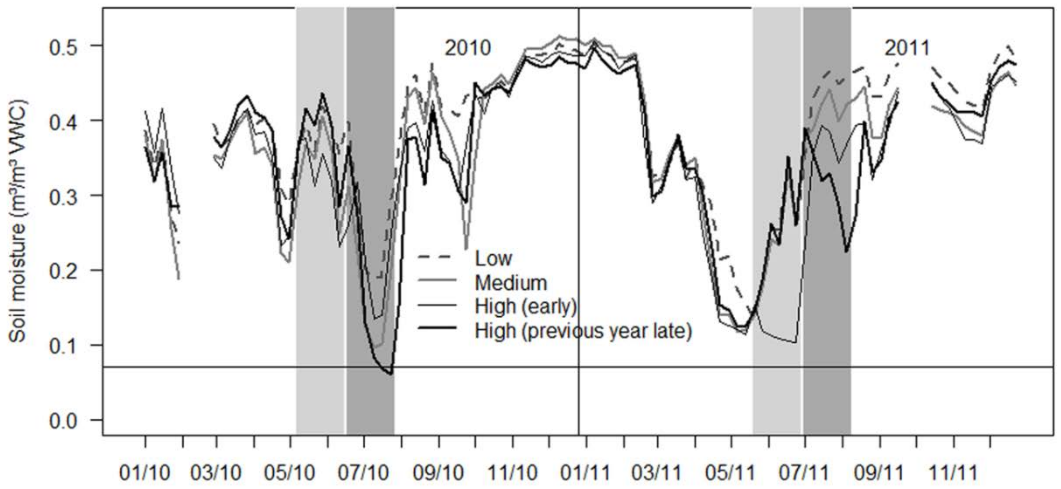


Figure 1: Soil moisture (-2 to -7 cm) under the precipitation variability treatments over the two consecutive study years (2010 and 2011). Data were collected from each treatment plot per precipitation block (n = 5) in 1-h intervals. Three days mean soil moisture (calculated from each 1-h mean) per treatment are shown here. Grey shade indicates the period of early variability and dark grey shade indicates the period of late variability.

Warming treatment

According to seasonally differing temperature change during last 100 years (Deutscher Wetterdienst 2015, see also in Table 1) and current projections (Jacob 2009; IPCC 2013), the warming treatments were carried out during the winter months October – March (Winter warming) or summer months April – September (Summer warming). The temperatures were increased using IR-overhead heating lamps (IOT/90 250W Elstein, Northeim, Germany) at a height of 0.8 m, theoretically resulting in 60 W plot^{-1}. IR-heating lamps were raised to 1 m if tall grasses reached 80 cm. Plots with no warming treatment were equipped with dummy lamps. Air temperature at +5cm (with radiation shield) and soil temperature at -2cm were recorded on an hourly basis by thermistors (B57863-106 S302-F40, EPCOS AG, München, Germany) in one warmed and one not warmed plot per precipitation block. All data collection took place in the center of each plot directly below the lamp to avoid potential edge effects created by temperature gradients with distance from the lamp. The air temperature at +5 cm above ground was raised on average by 1.0 °C in winter warming plots and by 1.3 °C in summer warming plots during the manipulation periods (Table 1). Soil temperature at -2 cm was raised by 1.4 °C in winter warming plots and by 1.1 °C in summer warming plots, respectively. Winter warming reduced the snow cover by more than one half in winter warming plots (Supporting information Fig. S1).

Table 1: Mean air (+5 cm) and soil (-2 cm) temperatures in ambient vs. warming treatment for summers and winters (2010-2011) and the respective temperature change due to warming. Given are mean seasonal temperature values and standard deviation.

	Ambient (mean ± SD °C)	Warming (mean ± SD °C)	Warming realized (mean °C)	Historical warming[1] (mean °C)
Air temperature				
Winter 2009/2010	1.5 ± 5.6	2.5 ± 5.8	+1.0	+1.1
Winter 2010/2011	2.2 ± 3.8	3.2 ± 4.5	+1.0	
Summer 2010	14.8 ± 6.0	15.9 ± 5.8	+1.1	+1.1
Summer 2011	14.2 ± 6.4	15.6 ± 6.3	+1.4	
Soil temperature				
Winter 2009/2010	2.6 ± 3.7	4.0 ± 4.2	+1.4	
Winter 2010/2011	2.5 ± 2.9	3.8 ± 3.6	+1.3	
Summer 2010	14.4 ± 4.7	15.5 ± 4.4	+1.1	
Summer 2011	14.0 ± 5.0	15.0 ± 4.7	+1.0	

[1] Source: Deutscher Wetterdienst (2015) for the period of 1881-2014.

Winter rain

The winter rain treatment was carried out during November to February. The control plots received ambient precipitation throughout the whole winter. In addition to ambient precipitation, the winter rain plots received 15 l/m^2 (=mm) water for four consecutive months in mid November, mid December, mid January and mid February (60 l/m^2 in total), which corresponds to an additional 60 mm precipitation in each winter rain plot. Winter rain addition increased the soil moisture in both years (at least 8 to 10 weeks), but had no effect on the snow cover (Fig. S2).

Flowering phenology, cover and biomass

The onset of flowering was recorded weekly in 2010 (starting in March until end of June) and in 2011 (starting in April until end of June) for 45 plant species in the central square meter of each plot in order to minimize edge effects. The onset of flowering was considered as the first day of an open flower with visible anthers. The documentation of the onset of flowering was conducted until the biomass harvest at the peak growing season in both years (21 June 2010 and 4 July 2011, respectively). Standing plant biomass of all species was harvested from the core of each plot (0.1m^2 rectangle). Collected samples were sorted to species and dried to a constant weight at 75 °C and weighed (Ohaus NavigatorTM, Ohaus Corporation, Parsippany, NJ, USA; accuracy±0.01 g). Species-specific plant cover (%) was visually estimated by the same two persons before each harvest. As the recording of the onset of flowering stopped with the biomass harvests, which corresponds with the end of the spring drought (= "$\text{high}_{\text{early}}$" treatment), the impact of the previous years 2009 and 2010, respectively, (such as ecological stress memory effects, see Walter *et al.* 2013) were examined for the summer drought treatment (= "$\text{high}_{\text{previous year late}}$" treatment). According to Jäger (2011), plants were classified as early flowering species, if their onset of flowering started before/within May and all plants flowering after May were classified as late flowering species. However, four early flowering species (according to Jäger 2011) were adjusted as late flowering species (*Crepis biennis*, *Dactylis glomerata*, *Poa pratensis* and *Trifolium repens*) as the onset of their flowering did not start before May in our experiment. Similarly, two late flowering species (according to Jäger 2011) were adjusted as early flowering species (*Leontodon hispidus* and *Vicia cracca*) as the onset of their flowering started before May in our experiment (Table S1).

Statistical analysis

Analysis of variance (ANOVA) combined with linear mixed effect models was applied to test for the main effects of the factors; warming (levels: "winter warming", "summer warming", "control"), growing season precipitation variability (levels: "low", "medium", "$\text{high}_{\text{early}}$", "$\text{high}_{\text{previous year late}}$"), winter rain (levels: yes or no), year (level: 2010 and 2011) and the interaction of each factor with the factor "flowering" (levels: "early flowering" or "late flowering" groups) on the onset, plant cover and biomass at the community level. The nested design with the warming and the winter rain treatments nested within the precipitation variability treatments was accounted for by random effects. Plot wise (n = 5 for each factor combination) average day of onset of flowering was used for community analysis. Average day of onset for early and late flowering community was calculated from the responses of all species within a plot. Plot replication was used as random factor.

In addition, plant species were analyzed separately if the onset of flowering of a species was recorded at least three times per year in any single treatment (n = 3) and also in the respective control (n = 3). Based on this criterion, 13 early flowering species and 12 late flowering species were suitable for species-specific data analysis (Table 4). ANOVA combined with linear mixed effect models were applied to test the onset of flowering at the species level. Replication and year (if no significant variation between years) were used as random factors. Furthermore, regression analysis between biomass (gm^{-2}) and onset (day of the year) of flowering species was conducted separately under control and warming treatments (as warming treatments had significant effects on community-average flowering onset) to see whether phenological shift could explain any changes in biomass responses. Biomass (not cover) was selected for this analysis as community-average biomass of late flowering species significantly differed under warming treatments compared to control (cover responses of both early and late species and biomass of only early species did not significantly differ under warming compared to control, see results for details). Regression analysis was done with linear least-squares regression (function 'lm'). Relative treatment effects on biomass (gm^{-2}) and onset (day of the year) of each species was also calculated (Δ = (treatment – Control)/Control.

Homogeneous groups of factor combinations were identified by Tukey's HSD post hoc comparisons. Level of significance was set to $p < 0.05$. LMEMs were conducted with the function 'lme' and Tukey HSD post hoc comparisons by the function 'glht'. Plot wise average onset of flowering (of the year) was calculated by the function 'aggregate'. The five packages multcomp (Simultaneous Inference in General Parametric Models, version:1.3-2), lme4 (Linear mixed-effects models using Eigen and S4, version: 1.0-6), sciplot (Scientific Graphing Functions for Factorial Designs, version: 1.1-0), Rmisc (Ryan miscellaneous, version: 1.5) and ggplot2 (An implementation of the Grammar of Graphics, version: 1.0.0) were used for data analysis and graph preparation in R version 3.2.1 (R Core Team, 2015).

Results

Effects of seasonal climate warming

At the community level, winter warming significantly advanced the onset of flowering in both years 2010 and 2011 (p = 0.0001, by 3.1 days; p < 0.0001, by 3.1 days, respectively, Fig. 2, Table 2). Summer warming significantly advanced the onset of flowering in the overall grassland community in 2010 (p = 0.0001, by 2.7 days), but not in 2011 (Fig. 2). The advanced phenological shift in response to seasonal warming was mainly caused by early flowering species. Winter warming significantly advanced the onset of early flowering species by 3.8 days in 2010 and by 6.1 days in 2011 (p < 0.0001 in both year) (Fig. 2). Winter warming had no statistically significant effect on the onset of late flowering species in both years. On the other hand, summer warming advanced the onset of early flowering species by 3.5 days (p = 0.0004) and of late flowering species by 2.8 days (p = 0.0012) in 2010, but not in 2011.

The warming treatments significantly altered plant cover and biomass production of early vs. late flowering species (Warming × Flowering (early/late); Cover, F=5.1, p=0.0095; Biomass, F=8.7, p=0.0002, Table S3). Winter warming significantly increased the biomass production (66%) and tended to increase the cover (13%) of late flowering species compared to control while early flowering species showed neither a significant change in cover nor in biomass in response to winter warming (Fig. 3). Summer warming showed no significant change in biomass or cover for any of the two species groups compared to control (Fig. 3).

Table 2: ANOVA results of the phenological community-average responses of the studied temperate grassland community to seasonal climatic treatments. Significant results ($p < 0.05$) are marked bold. Flowering species: early or late; early flowering species if their onset start before/within May and late flowering species if their onset start after May.

Factors	numDF	denDF	F-value	p-value
Warming (control, winter, summer)	**2**	**64**	**9.5**	**0.0002**
Precipitation variability (4 treatments)	3	64	0.8	0.4792
Winter rain (no, yes)	1	64	0.9	0.3455
Year (2010, 2011)	**1**	**190**	**135.1**	**<.0001**
Warming × Flowering (early or late)	**2**	**190**	**6.2**	**0.0024**
Precipitation × Flowering	3	190	1.3	0.2690
Winter rain × Flowering	1	190	1.0	0.3283
Precipitation × Warming	6	64	0.7	0.6869
Precipitation × Winter rain	3	64	1.2	0.3240
Precipitation × Warming × Flowering	6	190	1.7	0.1165
Precipitation × Winter rain × Flowering	3	190	0.3	0.8308
Warming × Year	**2**	**190**	**3.2**	**0.0441**
Precipitation × Year	3	190	1.4	0.2523
Winter rain × Year	1	190	0.0	0.8538
Flowering × Year	**1**	**190**	**97.6**	**<.0001**
Warming × Flowering × Year	2	190	2.4	0.0949
Precipitation × Flowering × Year	3	190	0.4	0.7772
Winter rain × Flowering × Year	1	190	0.6	0.4355
Precipitation × Warming × Year	6	190	1.5	0.1799
Precipitation × Winter rain × Year	3	190	0.5	0.6979
Precipitation × Warming × Flowering × Year	6	190	0.2	0.9735
Precipitation × Winter rain × Flowering × Year	3	190	0.1	0.9402

At the species level, warming effects on the onset of flowering were species-specific (Table 3). Winter warming significantly advanced the onset of three early flowering species in 2010 (*Alopecurus pratensis*, 5.3 days; *Taraxacum officinale*, 10.4 days; *Veronica arvensis*, 13.4 days) and five early flowering species in 2011 (*Alopecurus pratensis,* 10.0 days; *Anthoxanthum odoratum,* 8.8 days; *Ranunculus acris*, 5.6 days; *Trifolium dubium*, 12.3 days; *Vicia sepium*, 28.3 days; Table 3). Summer warming significantly advanced the onset of one early flowering species (*Veronica arvensis*, 10.1 days) in 2010, and another early flowering species (*A. pratensis*, 4.5 days) in 2011. Contrarily, onset of only one late flowering species (*Holcus lanatus*) was significantly advanced by winter warming in 2011 (10.3 days) and by summer warming in 2010 (6.6 days). No single-species effects of winter warming in 2010 and summer warming in 2011 were detected on any late flowering species. No significant correlations between single-species biomass (gm^{-2}) and onset (day of the year) of both early and late flowering species were detected under any seasonal warming treatments (regression data not shown, relative treatment effects on single-species biomass are shown in table 3).

Effects of additional winter rain and growing season precipitation variability

At the community level across species, additional winter rain and growing season precipitation variability had no effect on the onset of flowering in any of the two years (Winter rain × Year, p = 0.8538; Precipitation variability × Year, p = 0.2523; Table 3; Years did not differ for winter rain and precipitation variability, therefore average onset over two years is shown in the Fig. 4). Additional winter rain had no significant single and interaction effects on cover and biomass for both early and late flowering species compared to control (Table S3 and Fig. S3). Precipitation also had no significant effects on biomass for early and late flowering species compared to control (Table S3 and Fig. S4).

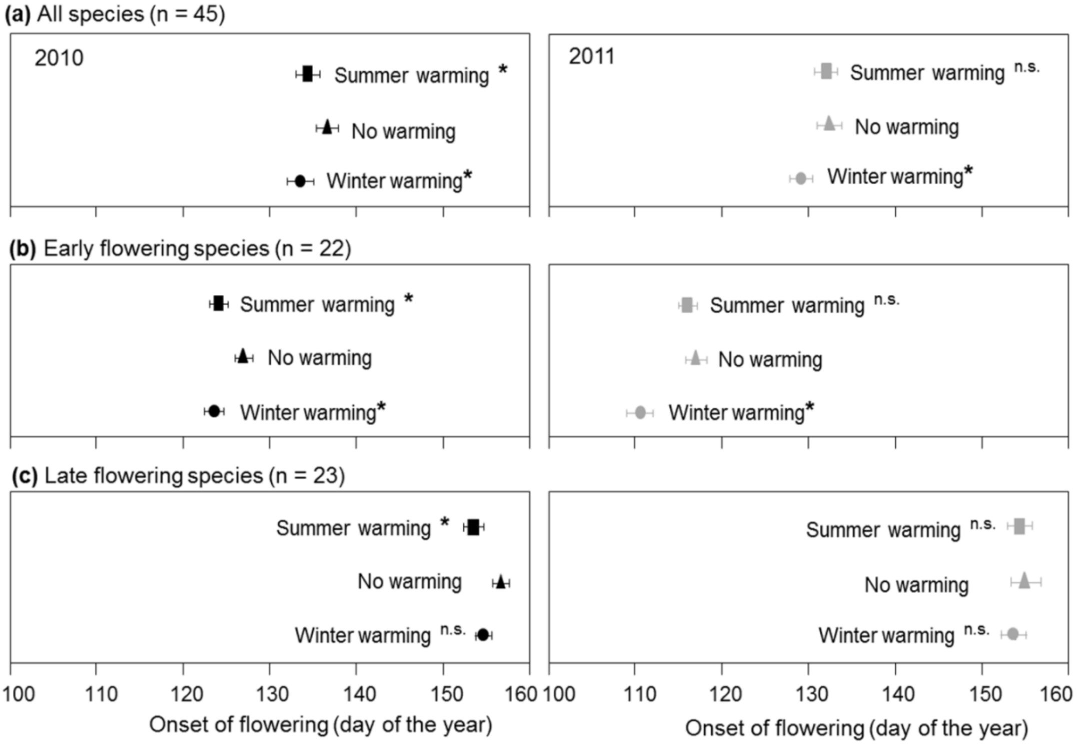
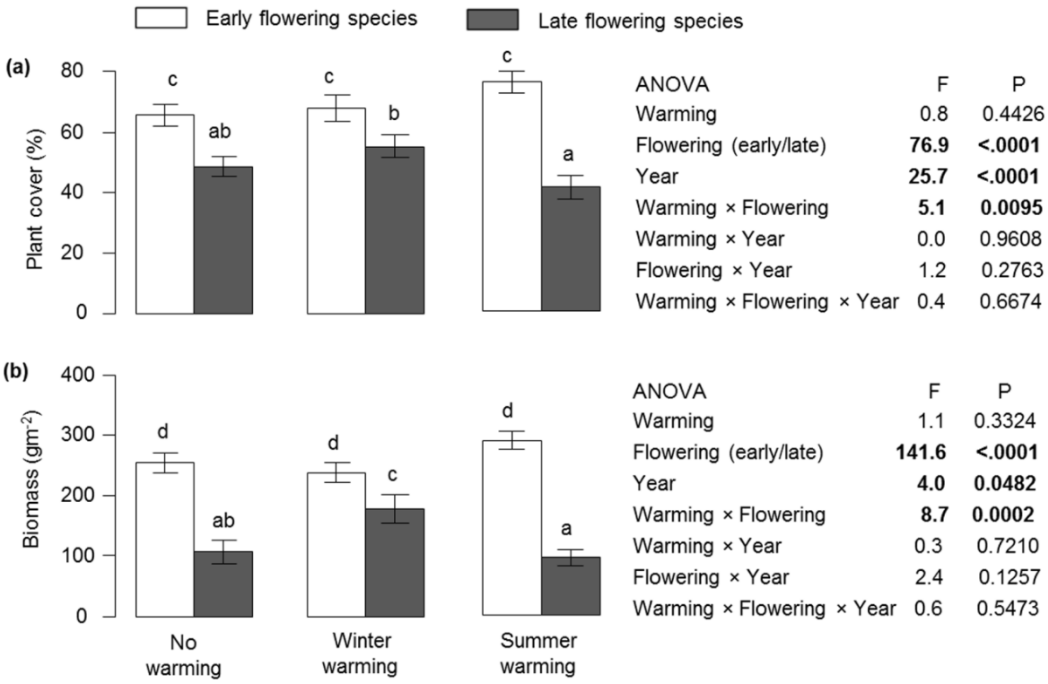


Figure 2: Community-average onset of flowering of a temperate grassland community under winter and summer warming (a) all species, (b) early flowering species (c) late flowering species. Average onsets of flowering and slandered error per plot are shown here (n = 5 per treatment). Significant treatment effects compared to control are marked by asterisks ($p < 0.05$). “n.s.”- not significant.

However, a few species-specific responses were detected (average onset over two years are shown in the Table 4). Addition of winter rain advanced the onset of one early (T. dubium, 7.2 days) and one late (H. lanatus, 3.2 days) flowering species. The low precipitation variability treatment also advanced the onset of the same early flowering species (T. dubium, 10.7 days). No significant species-specific responses were detected under the spring drought (highearly precipitation variability) treatment. However, the summer drought (highprevious year late precipitation variability) treatment delayed onset of one early flowering species (Veronica chamaedrys, 14.3 days) and advanced onset of one early flowering (T. dubium, 9.2 days) and one late flowering species (Dactylis glomerata, 3.6 days).



ANOVA	F	P
Warming	0.8	0.4426
Flowering (early/late)	**76.9**	**<.0001**
Year	**25.7**	**<.0001**
Warming × Flowering	**5.1**	**0.0095**
Warming × Year	0.0	0.9608
Flowering × Year	1.2	0.2763
Warming × Flowering × Year	0.4	0.6674

ANOVA	F	P
Warming	1.1	0.3324
Flowering (early/late)	**141.6**	**<.0001**
Year	**4.0**	**0.0482**
Warming × Flowering	**8.7**	**0.0002**
Warming × Year	0.3	0.7210
Flowering × Year	2.4	0.1257
Warming × Flowering × Year	0.6	0.5473

Figure 3: Responses of early vs late flowering community (a) plant cover (%) and (b) biomass (gm^{-2}) at peak growing season under warming treatments. Average community responses and standard error per plot are shown (n = 5 / treatment). Significant results ($p < 0.05$) are marked bold.

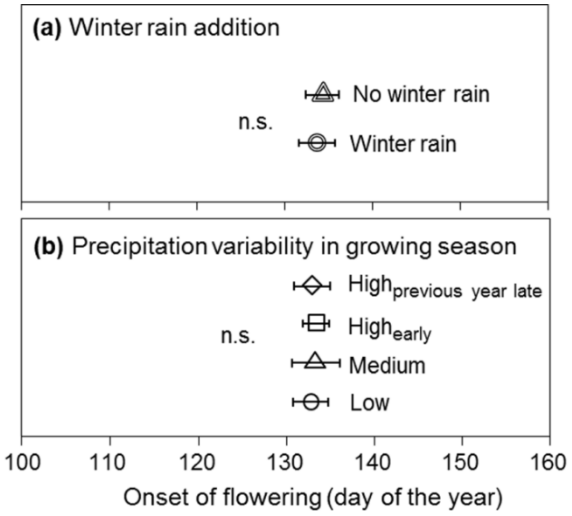


Figure 4: Community-average onset of flowering of a temperate grassland community under (a) winter rain addition and (b) four precipitation variability treatments during the growing season. Average onsets of flowering and standard error per plot are shown (n = 5 per treatment). “n.s.”- not significant.

Table 3: Shift in onset of flowering and biomass responses of the single early and late flowering species to the seasonal climate treatments. Relative treatment effects on biomass are given in the parentheses (if species are present in both control and treatment plots). Effects of seasonal warming on the onset of flowering differed between years (warming × year, $p = 0.0441$, Table 3). Therefore, responses to warming treatment are shown separately for both years. Averages over both years (2010 and 2011) are shown for the precipitation treatments as responses did not differ between years (Table 3). Positive (+) values indicate delay and negative (-) values indicate earlier onset of flowering date in days compared to control. Significant (ANOVA, $p < 0.05$) effects between control (the mid variability treatment for the precipitation variability treatments) and the treatments are marked bold for the flowering onset. For biomass, positive (+) values indicate relative increase and negative (-) values indicate relative decrease by treatments compared to control (Relative responses = (treatment –Control)/Control). “NA” = if the onset of flowering was not observed at least three times in the specific treatment or in the respective control plot.

species	Winter warming		Summer warming		Winter rain	Low precipitation variability	High$_{\text{early}}$ precipitation variability	High$_{\text{previous year late}}$ precipitation variability
	Year 2010	Year 2011	Year 2010	Year 2011	Both years	Both years	Both years	Both years
Early flowering species								
Alopecurus pratensis	**-5.3** (-0.15)	**-10.0** (-0.14)	-1.8 (+0.8)	**-4.5** (+0.23)	-1.2 (+0.16)	+0.5 (+0.01)	+0.2 (-0.14)	+0.3 (-0.05)
Anthoxanthum odoratum	-2.2 (-0.16)	**-8.8** (-0.16)	-0.7 (-0.27)	-0.2 (-0.23)	-1.8 (-0.40)	-0.7 (+0.42)	-1.9 (+0.41)	+0.5 (-0.43)
Bellis perennis	-6.9 (+2.34)	-3.5 (0.00)	-8.0 (-0.08)	+3.6 (0.00)	-5.1 (+0.21)	-1.0 (-0.03)	+3.0 (-0.02)	+0.5 (-0.10)
Cerastium holosteoides	-4.1 (+2.76)	-1.4 (-0.21)	-5.3 (+0.46)	-0.8 (-0.49)	+0.7 (-0.07)	+0.9 (-0.15)	-0.5 (-0.01)	-2.4 (-0.15)
Plantago lanceolata	+0.7 (+0.24)	-4.2 (+0.92)	-3.2 (+0.17)	+6.7 (+0.33)	+0.6 (-0.06)	-3.7 (+0.65)	-4.9 (+1.62)	-3.5 (+1.20)
Ranunculus acris	-3.8 (-0.13)	**-5.6** (-0.63)	-5.4 (+1.22)	+1.4 (-0.70)	-0.3 (-0.42)	-5.7 (-0.19)	-0.7 (+0.06)	-2.3 (+0.83)
Rumex acetosa	-0.2 (-0.08)	-5.3 (+0.22)	-0.3 (-0.22)	0.0 (+0.12)	+0.2 (-0.02)	-1.2 (+0.11)	+0.6 (-0.15)	-0.4 (-0.16)
Taraxacum officinale	**-10.4** (+1.20)	-5.1 (+0.67)	-5.2 (+7.17)	+1.0 (+3.62)	+0.7 (+0.35)	+1.7 (-0.65)	-0.1 (+1.90)	-0.5 (-0.02)
Trifolium dubium	-4.4 (-0.46)	**-12.3** (0.00)	-10.3 (+1.42)	NA	**-7.2** (+0.04)	**-10.5** (-0.72)	-8.4 (-0.63)	**-9.2** (+0.82)
Veronica arvensis	**-13.4** (-0.40)	NA	**-10.1** (0.00)	NA	-2.2 (-0.69)	+2.7 (0.00)	+3.7 (-0.76)	+3.8 (+2.33)
Veronica chamaedrys	NA	-2.8 (0.00)	NA	+7.2 (0.00)	+1.0 (0.00)	+0.3 (0.00)	+7.6 (0.00)	**+14.3** (0.00)
Veronica serpyllifolia	-10.0 (0.00)	NA	-7.7 (0.00)	NA	-10.5 (0.00)	-4.3 (0.00)	+9.7 (0.00)	+0.3 (0.00)
Vicia sepium	-7.8 (+2.48)	**-28.3** (+89.9)	NA (+32.4)	-22.5 (+6.75)	-7.5 (+3.83)	+11.9 (-0.53)	NA	+20.5 (-0.64)
Late flowering species								
Arrhenatherum elatius	-0.9 (+1.77)	+2.1 (+0.62)	-1.9 (+0.13)	-1.0 (+0.42)	-1.7 (+0.17)	+1.5 (+0.49)	-1.2 (+0.31)	+0.8 (+0.11)
Centaurea jacea	NA (-0.69)	-1.2 (+2.03)	NA (-0.98)	+0.4 (-0.91)	-1.4 (-0.92)	+2.7 (+3.67)	+4.6 (+5.18)	-4.5 (+8.72)
Dactylis glomerata	NA (+1.13)	-3.0 (+0.81)	NA (-0.19)	-11.8 (-0.13)	+0.2 (+0.87)	-1.2 (-0.18)	-4.6 (-0.75)	**-3.6** (-0.02)
Festuca rubra	NA (-0.25)	+3.0 (+0.88)	NA (-0.74)	+2.3 (-0.45)	+2.9 (-0.71)	-3.4 (+0.74)	-0.7 (-0.32)	-3.6 (-0.23)
Galium album	0.0 (+0.79)	+2.3 (+14.14)	-5.1 (+8.6)	-1.1 (+15.91)	-0.1 (+0.23)	-2.1 (+3.99)	-2.5 (+0.95)	+1.3 (-0.76)
Holcus lanatus	-1.3 (+0.49)	**-10.3** (+0.86)	**-6.6** (-0.08)	-3.9 (+0.12)	**-3.2** (-0.03)	+0.3 (+0.25)	-1.5 (+0.13)	-0.2 (-0.17)
Hypochoeris radicata	NA (+0.88)	+6.5 (-0.32)	NA (-0.89)	+8.7 (-0.95)	+9.0 (-0.74)	-1.2 (+4.84)	+1.8 (-0.74)	-1.7 (+0.41)
Leucanthemum ircutianum	0.0 (0.00)	NA	NA (+0.86)	NA	-2.4 (-0.55)	NA	-3.8 (+3.88)	-8.8 (+6.51)
Phleum pratense	NA (-0.10)	+0.1 (+1.38)	NA (-0.18)	-0.3 (+5.89)	-1.6 (+0.40)	+2.5 (+2.31)	+3.5 (-0.32)	-0.3 (-0.33)
Poa pratensis	-2.6 (+0.16)	0.0 (+0.91)	-1.8 (+0.48)	-1.9 (+0.45)	-0.7 (+0.19)	-1.5 (+0.24)	-2.9 (-0.21)	-1.2 (-0.01)
Poa trivialis	NA (+1.99)	NA (+9.40)	NA (-0.21)	NA	NA (+1.23)	-0.3 (+0.21)	-5.8 (-0.46)	NA +1.99)
Trifolium pratense	-6.2 (-0.14)	-4.2 (+2.83)	-5.0 (-0.11)	NA (-0.29)	-9.4 (+0.73)	+3.9 (-0.55)	+0.8 (-0.72)	-3.3 (-0.19)

Discussion

Effects of seasonal climate warming on onset of flowering

We confirmed the first hypothesis that winter and summer warming advance the onset of flowering of plant species in a species-rich temperate grassland ecosystem. On average over two years, winter warming (air, +1.0°C; soil, +1.4°C) advanced the onset of flowering of the temperate grassland community by 3.1 days while summer warming (air, +1.3°C; soil, +1.1°C) advanced flowering by only 1.5 days.

The experimental data confirm the expectation that warming advances the flowering phenology of grassland species. As in our study seasonal warming was separately affected, we could show that the phenological shift due to winter warming was stronger than the one in response to summer warming. This indicates specific phenological sensitivity of grassland species to seasonal warming. Most of the existing studies suggest that flowering phenology is highly sensitive to the average monthly temperature of the preceding two months and the month of flowering onset (Chmielewski & Rötzer, 2001; Menzel *et al.*, 2006; Nagy *et al.*, 2013). The summer warming treatment was carried out during the preceding two months (April-May) and also during the month of peak flowering onset (June) of the late-flowering species in the present study. The mean temperature enhancement for the manipulation period was similar (1.2°C) both in winter and summer, but winter warming advanced flowering phenology (by 3.1 days) more than summer warming (by 1.5 days). Earlier flowering due to temperature increase was reported for many plant species and regions. In temperate Europe, it is reported that plant phenology is shifting -2 to -5 days per 1°C increase (Chmielewski & Rötzer, 2001; Menzel *et al.*, 2006). Interestingly, flowering phenology of the studied semi-natural temperate grassland community was more responsive to 1°C temperature increase in winter (October – March) than to 1°C increase in summer (April – September). Furthermore, a recent study shows that global warming effects on the phenology of spring leaf unfolding of dominant European tree species has significantly decreased from 1980 to 2013 (Fu *et al.*, 2015). Phenological responses of European tree species are not directly comparable with European grassland species. However, our results indicate that only summer warming has little to no effect on flowering phenology but winter warming can be strongly influential in temperate grassland ecosystem.

The hypothesis that the onset of early flowering species will be advanced by winter warming and the onset of late flowering species will be advanced by summer warming, was only partly supported by the present study. In accordance with our expectations, winter warming significantly advanced the onset of early flowering species in both years (2010, by 3.8 days; 2011, by 6.1 days), but had no effect on late flowering species in any of the two years. Winter warming effects in both years is related to the direct impacts of raising air and soil temperature during the months preceding (February and March) onset of early flowering species (as indicated by 1.2°C warming compared to control, see table 1 & 2). The higher winter warming effects on early flowering species in 2011 compared to 2010 could potentially be due to the differences in the growing season start date. Higher average air temperature during winter 2011 compared to 2010 (Table 1) indicate an earlier start to the growing season in 2011. Onset of flowering of the early flowering species started (irrespective of treatment) about 10 days earlier in 2011 compared to 2010. Summer warming advanced the onset of early (by 3.5 days) and late (by 2.8 days) flowering species only in 2010 but not in 2011. The missing effects in 2011 for the summer warming is probably also due to the earlier start of the growing season in that year. At least the majority of early flowering species had already started flowering in March which is before the summer warming even started in April 2011.

Correlation between single-species biomass and flowering onset of early and late flowering species was not statistically significant. However, community-average cover/biomass data shows that winter warming significantly increases the biomass of late flowering species, while winter warming has no effects on cover/biomass of early flowering species compared to control. This indicated that the (smaller) response in phenology of the late flowering species might simply be an artefact of increased biomass of those species rather than true changes in phenology, while the phenological response of the early flowering species could not be explained by increased cover/biomass. In sum, our main finding of the early flowering species being more responsive in their phenology is obviously a true phenological effect.

Taken together, we conclude that advanced flowering phenology in response to gradual warming in a temperate grassland ecosystem is mainly due to the high phenological sensitivity of early flowering species to winter warming. Species-specific flowering onset demonstrates that one third of the early flowering species react significantly to seasonal warming. This means that even within the early flowering community there are divergences. The phenological advance of early flowering species is in accordance with previous findings in a North American natural prairie (Sherry *et al.*, 2007). Still, the direction of the observed phenological shift of late flowering species towards earlier flowering does not match the findings of Sherry *et al.* (2007) who found warming delayed the onset of late flowering species. This indicates that phenological responses depend on the regional climate and ecosystem. Temperate managed grasslands (e.g. hay meadows) cannot be directly compared to continental natural prairies because of the differences in climate, length of growing season, plant composition and importantly because of the substantially different management activities (mowing, which means synchronized extraction of above ground biomass).

We investigated the onset of flowering of a typical temperate European managed grassland community (hay meadow), where many plant species are flowering before June due to their adaptation to seasonality and land use regimes (Table S1). The observed semi-natural plant community (n = 45) and its species are representative for European managed grassland (hay meadows). Therefore, we suggest that the detected community level phenological responses are representative for temperate grassland ecosystems across Europe.

Effects of winter rain addition and growing season precipitation variability on onset of flowering

The present experimental study provides evidence that average flowering onset of a species-rich temperate grassland community is not impacted by winter rain addition and growing season precipitation variability. However, at the species level, a few responded significantly to the precipitation treatments. Such species-specific responses are in accordance with previous findings (e.g. Jentsch *et al.* 2009; Nagy *et al.* 2013). Most of the existing studies, however, involved single or only a few species and reported species-specific phenological shifts under sudden changes in moisture regimes. The present study showed no phenological shift at the community level potentially because the responses of a semi-natural grassland with 45 species were considered, indicating that biodiversity can increase the resistance of grassland phenology to precipitation variability *sensu* the insurance hypothesis (Yachi & Loreau, 1999). A recent study over 46 grassland biodiversity experiments across Europe and North America showed that species richness increases resistance of ecosystem productivity to extreme precipitation events (Isbell *et al.*, 2015). The present study does not provide a generalization across a biodiversity gradient, but at least shows that flowering phenology of a species-rich grassland community is stable under modified precipitation amount and variability.

Implication of climate change driven phenological shifts to plant functioning

Phenology of early flowering temperate grassland species was advanced by winter warming more than by summer warming, while late flowering species were generally less sensitive to seasonal warming. Our results show that in temperate grassland ecosystems seasonal modifications in warming, mainly winter warming, might lengthen the flowering period of early flowering species compared to late flowering species. This finding indicates that winter warming can decrease temporal overlap, increase phenological complementarity, and potentially decrease competition for resources and pollinators between early and late flowering species. On the other hand, winter warming can alter vegetation dynamics of early and late flowering species by changing physiological and morphological activities of early flowering species stronger than that of late flowering species. It has been suggested that earlier phenological development can alter reproductive fitness of plants and can play an important role in niche differentiation among coexisting species, this is because temperature dependent earlier phenology is related to earlier root growth, leaf expansion, seed maturation, higher plant N uptake and also success of exotic species

(McKane *et al.*, 1990; Santandreu & Lloret, 1999; Saavedra *et al.*, 2003; Wolkovich *et al.*, 2013). These changes could potentially trigger further shifts in seasonality of other ecosystem processes (e.g. plant-pollinator interactions).

If pollinators do not adapt their life stages with ongoing global warming and plant phenological shift, this would negatively impact reproduction of insect-pollinated plants due to phenological mismatch. For example, Liu *et al.* (2011) reported that warming increased larval density during the peak growing season and reduced gentian plant (*Gentiana formosa*) reproduction in an Tibetan alpine meadow. There is evidence for recent declines in pollinators globally (Potts *et al.*, 2010) and parallel declines in the plants that rely upon them (at least in western Europe, see Biesmeijer *et al.* 2006). It is suggested that 17% to 50% of all pollinator species suffer a disruption of food supply if phenology of plants shift –10 to –20 days (Memmott *et al.*, 2007). Our results indicate that flowering onset of the studied grassland ecosystem might shift –6.7 to –12.4 days by winter warming and –3.3 to –6.0 days by summer warming at the end of 21st century under the temperature scenario RCP8.5 if we assume a simple linear relation between warming and shift in phenology (see details in the table 4). Shifts as strong as this could extensively alter the plant-pollinator or plant-herbivore interactions.

Yet, as there was no significant evidence of community level phenological shifts under winter rain and modified growing season precipitation variability, we suggest that the overall plant-pollinator interaction in a species-rich temperate grassland will not change dramatically under intra-annual precipitation changes. However, the observed species-specific phenological shifts indicate that intra-annual precipitation changes have the potential to alter species dynamics, functioning and composition of temperate grassland.

Table 4: Predicted onset (first flowering date) of temperate grassland plants for 21st century. Onset shifts were predicted by linear extrapolation based on the observed results[1] and four global warming scenarios projected by IPCC (2013).

Predicted increase in temperature for 2081-2100 (IPCC, 2013)	Predicted shift in onset of grassland plants in 2081-2100 Winter warming	Summer warming
(a) All species (n = 45)		
0.3°C to 1.7°C (RCP2.6)	–0.8 to –4.4 days	–0.4 to –2.1 days
1.1°C to 2.6°C (RCP4.5)	–2.8 to –6.7 days	–1.4 to –3.3 days
1.4°C to 3.1°C (RCP6.0)	–3.6 to –8.0 days	–1.8 to –3.9 days
2.6°C to 4.8°C (RCP8.5)	–6.7 to –12.4 days	–3.3 to –6.0 days
(b) Early flowering species (n = 22)		
0.3°C to 1.7°C (RCP2.6)	–1.2 to –6.9 days	–0.6 to –3.3 days
1.1°C to 2.6°C (RCP4.5)	–4.5 to –10.6 days	–2.1 to –5.0 days
1.4°C to 3.1°C (RCP6.0)	–5.7 to –12.7 days	–2.7 to –5.9 days
2.6°C to 4.8°C (RCP8.5)	–10.6 to –19.6 days	–5.0 to –9.2 days

Note: [1] (a) All species: winter warming (air + soil = 1.2°C) shift –3.1 days, summer warming (air + soil = 1.2°C) shift –1.5 days; (b) Early flowering species: winter warming (1.2°C) shift –4.9 days and summer warming (1.2°C) shift –2.3 days, respectively. Responses of late flowering species are not given here as no significant phenological shift observed over two years.

Conclusions

Winter warming rather than summer warming emerged as the main driver for advancing flowering phenology of the studied semi-natural mesic temperate grassland community. Interestingly, early flowering species were more responsive to both, winter and summer warming, than late flowering species. Our findings emphasize the necessity of considering seasonality in climate change. The observed magnitudes of change have the potential to alter ecosystem functioning and community composition, e.g. by favouring early-flowering species or questioning plant-pollinator interactions. Flowering phenology of the studied temperate grassland ecosystem was largely unaffected by the realized precipitation changes, i.e. altered summer rainfall variability and increased winter rainfall.

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Supporting information

Fig. S1 Winter warming effects on snow cover (cm) over the two study years. Snow cover was measured manually on a weekly basis. Mean weakly snow cover height (cm) per plot (n = 5) are shown in the figure.

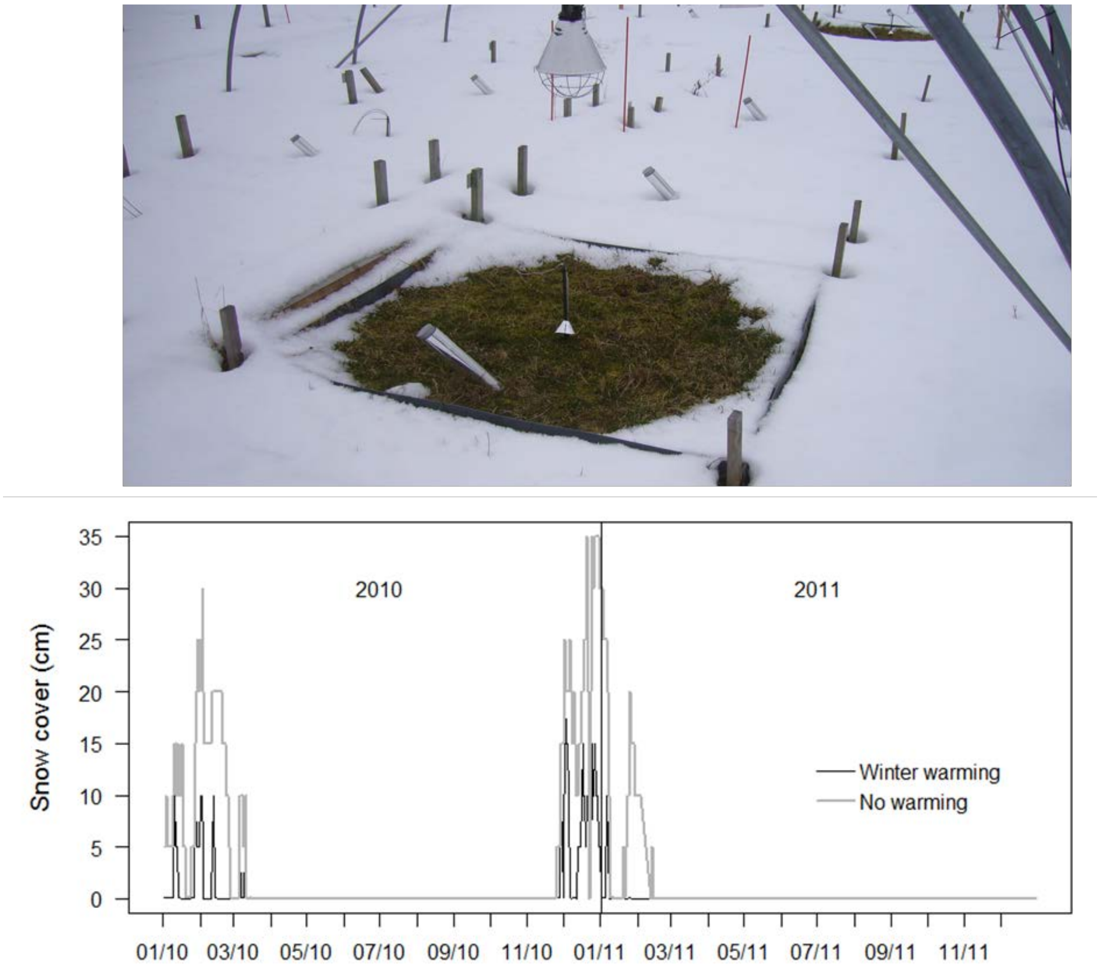


Fig. S2 Soil moisture and snow cover under winter rain addition over the two study years. Data were collected from each treatment plot per precipitation block (n = 5) in 1-h intervals. Three days mean soil moisture (calculated from each 1-h mean) per treatment are shown here. Snow cover was measured manually on a weekly basis.

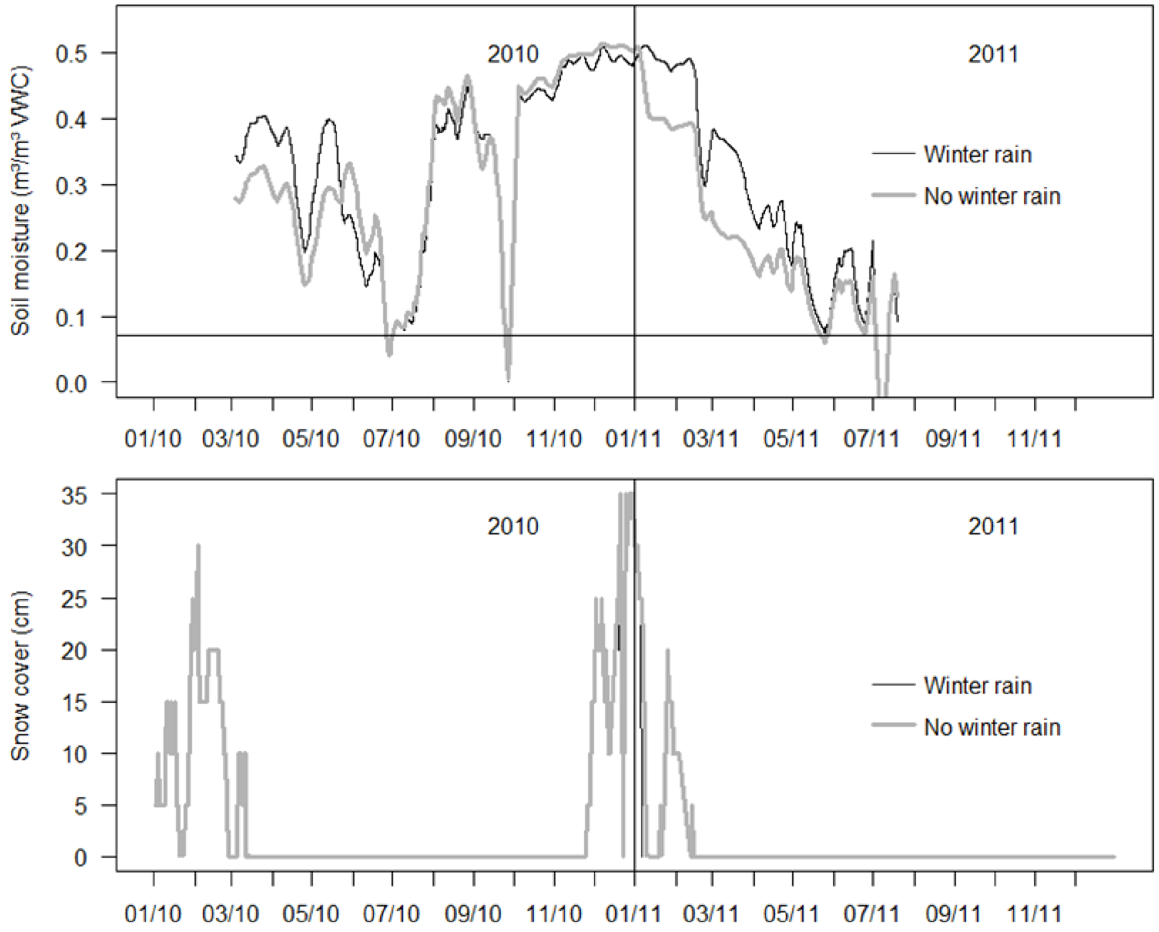
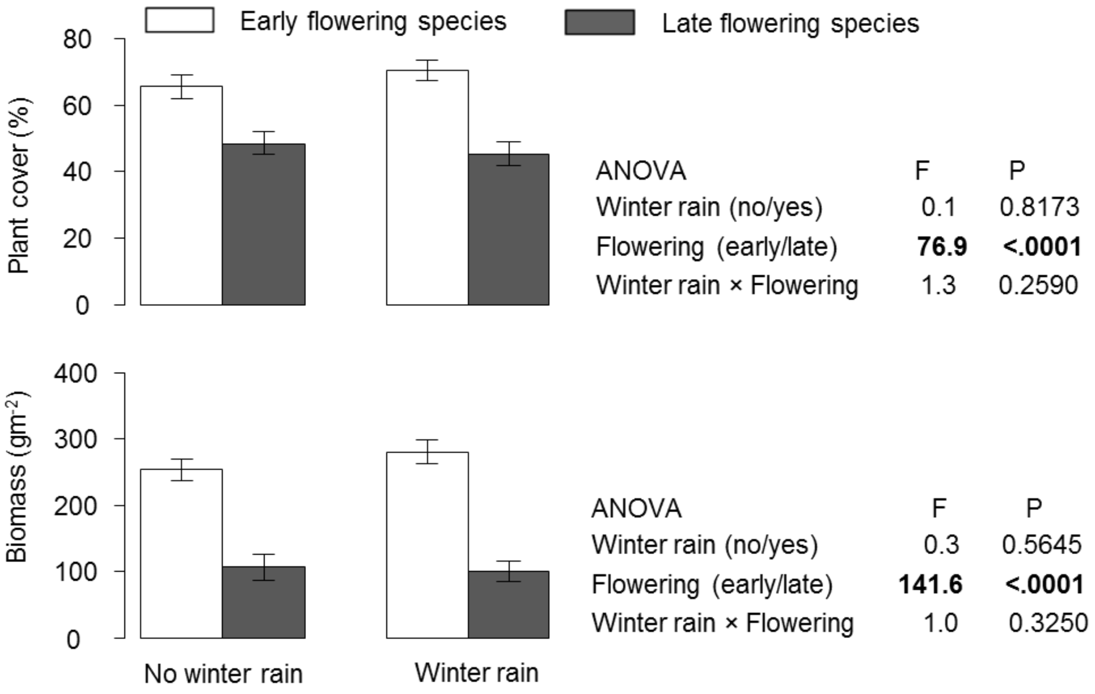


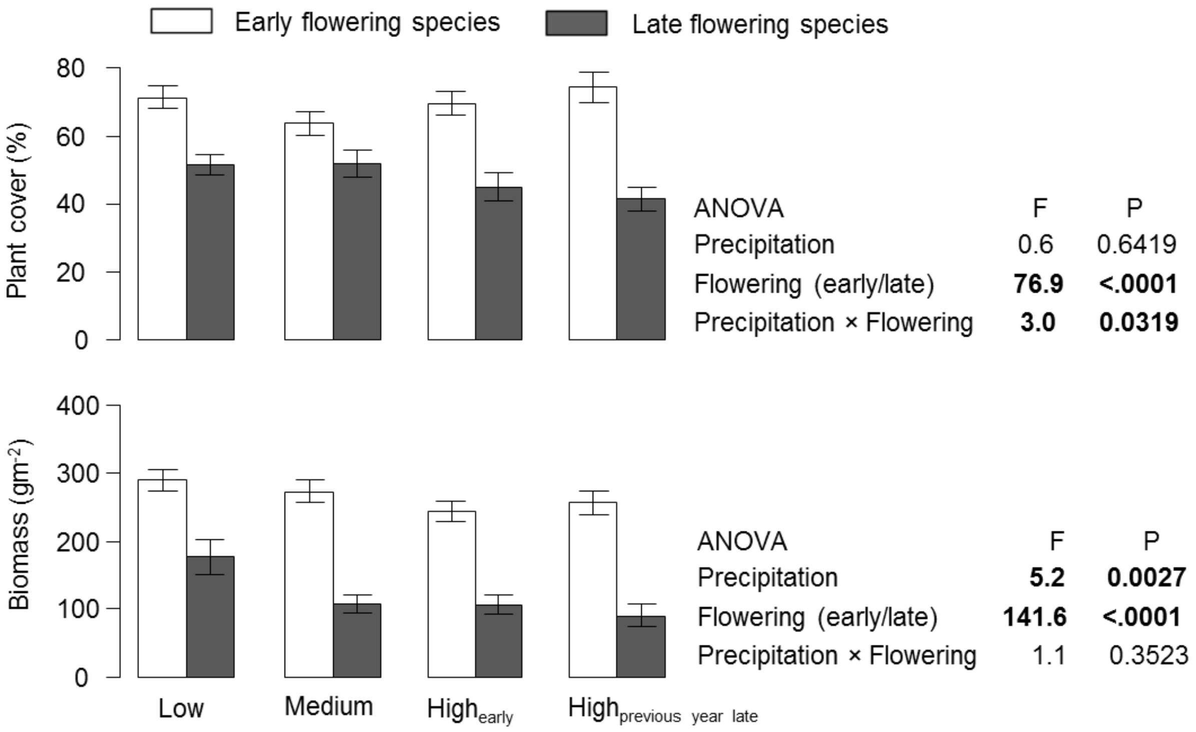
Fig. S3 Responses of early vs late flowering community (a) plant cover (%) and (b) biomass (gm^{-2}) at peak growing season under winter rain. Average community responses and standard error per plot are shown (n = 5 per treatment). Significant results ($p < 0.05$) are marked bold.



ANOVA	F	P
Winter rain (no/yes)	0.1	0.8173
Flowering (early/late)	**76.9**	**<.0001**
Winter rain × Flowering	1.3	0.2590

ANOVA	F	P
Winter rain (no/yes)	0.3	0.5645
Flowering (early/late)	**141.6**	**<.0001**
Winter rain × Flowering	1.0	0.3250

Fig. S4 Responses of early vs late flowering community (a) plant cover (%) and (b) biomass (gm^{-2}) at peak growing season under precipitation variability during growing season. Average community responses and standard error per plot are shown (n = 5 per treatment). Significant results ($p < 0.05$) are marked bold.



ANOVA	F	P
Precipitation	0.6	0.6419
Flowering (early/late)	**76.9**	**<.0001**
Precipitation × Flowering	**3.0**	**0.0319**

ANOVA	F	P
Precipitation	**5.2**	**0.0027**
Flowering (early/late)	**141.6**	**<.0001**
Precipitation × Flowering	1.1	0.3523

Table S1 List of all species checked for their flower phenology in the EVENT II experiment. Early flowering species - onset of flowering starting before/within May; late flowering species- onset of flowering starting after May. Four early flowering species (*Crepis biennis*, *Dactylis glomerata*, *Poa pratensis and Trifolium repens*; according to Jäger 2011) were adjusted as late flowering species as their onset of flowering did not start before May. Similarly, two late flowering species (*Leontodon hispidus* and *Vicia cracca*; according to Jäger 2011) were adjusted as early flowering species as their onset of flowering started before/within May.

Species	**Average onset of flowering irrespective of climate treatment (Day of the year)**	**SD**	**SE**	**Number of occurrence**	**Flowering length** (Jäger, 2011)
Early flowering species					
Alopecurus pratensis	116	7.1	0.5	200	May - Jun
Anthoxanthum odoratum	117	10.3	0.8	177	May - Jun
Bellis perennis	110	12.0	1.5	68	Jan - Apr
Cardamine pratensis	110	7.5	2.6	8	Apr - Jun
Carex pallescens	96	0.0	0.0	3	May - Jul
Cerastium holosteoides	125	9.7	0.8	141	Apr - Oct
Leontodon hispidus	131	11.8	4.5	7	Jun - Oct
Luzula campestris	111	8.3	2.2	14	Mar - Apr
Plantago lanceolata	131	15.2	1.5	102	May - Sep
Ranunculus acris	127	8.2	0.8	93	Apr - Sep
Ranunculus repens	140	13.7	5.6	6	May - Aug
Rumex acetosa	122	8.3	0.6	186	May - Jul
Taraxacum officinale	117	7.0	0.8	87	Apr - Jun
Trifolium dubium	145	8.9	1.5	33	May - Sep
Trisetum flavescens	149	25.5	18.0	2	May - Jun
Veronica arvensis	116	10.0	1.5	47	Mar - Jun
Veronica chamaedrys	109	10.3	1.7	35	Apr - Oct
Veronica serpyllifolia	129	11.8	2.9	17	May - Sep
Vicia angustifolia	131	17.8	5.1	12	May - Jul
Vicia cracca	119	4.0	2.3	3	Jun - Aug
Vicia sepium	133	13.0	2.5	27	May - Aug
Viola arvensis	117	NA	NA	1	Apr - Oct
Late flowering species					
Achillea millefolium	167	NA	NA	1	Jun - Oct
Agrostis capillaris	170	5.2	1.8	8	Jun - Jul
Arrhenatherum elatius	156	8.6	0.7	144	Jun - Jul
Centaurea jacea	161	6.1	1.1	31	Jun - Aug
Crepis biennis	151	2.7	1.2	5	May - Aug
Cynosurus cristatus	157	5.1	1.2	18	Jun - Jul
Dactylis glomerata	159	7.8	1.4	30	May - Jul
Festuca pratensis	162	3.4	1.3	7	Jun - Jul
Festuca rubra	160	5.5	1.0	29	Jun - Jul
Galium album	153	7.4	0.9	65	Jun - Sep
Holcus lanatus	153	8.7	0.8	109	Jun - Aug
Hypochaeris radicata	154	6.4	1.4	20	Jun - Sep
Knautia arvensis	148	8.9	2.4	14	Jun - Aug
Leucanthemum ircutianum	151	7.4	1.7	18	Jun - Oct
Lolium perenne	156	11.8	5.3	5	May - Oct
Lotus corniculatus	152	14.4	4.8	9	Jun - Aug
Phleum pratense	165	5.3	0.8	46	Jun - Aug
Poa pratensis	151	6.0	0.5	163	May - Jul
Poa trivialis	153	7.6	1.4	31	Jun - Jul
Rhinanthus minor	151	5.2	2.1	6	May - Sep
Trifolium pratense	147	14.0	1.8	62	Jun - Sep
Trifolium repens	155	9.6	3.2	9	May - Sep
Vicia hirsuta	144	12.2	4.6	7	Jun - Jul

Table S2 Coefficient of variation (CV) of daily precipitation amounts for the precipitation variability treatments over two study years (2010 and 2011). CV = standard deviation × daily mean - 1 × 100).

	Precipitation variability (CV)			
Year	Low	Medium	$\text{High}_{\text{early}}$	$\text{High}_{\text{previous year late}}$
2010	231	266	302	299
2011	217	251	353	295
Average	224	259	328	297

Table S3 ANOVA results of the community-average cover and biomass responses of the studied temperate grassland to seasonal climatic treatments. Significant results ($p < 0.05$) are marked bold. Flowering species: early or late; early flowering species if their onset start before/within May and late flowering species if their onset start after May.

Factors	numDF	denDF	Plant cover (%)		Biomass (gm^{-2})	
			F-value	p-value	F-value	p-value
Warming (control, winter, summer)	2	64	0.8	0.4426	1.1	0.3324
Flowering (early or late)	1	192	**76.9**	**<.0001**	**141.6**	**<.0001**
Precipitation variability (4 treatments)	3	64	0.6	0.6419	**5.2**	**0.0027**
Winter rain (no, yes)	1	64	0.1	0.8173	0.3	0.5645
Year (2010, 2011)	1	192	**25.7**	**<.0001**	**4.0**	**0.0482**
Warming × Flowering	2	192	**4.8**	**0.0095**	**8.7**	**0.0002**
Precipitation × Flowering	3	192	**3.0**	**0.0319**	1.1	0.3523
Winter rain × Flowering	1	192	1.3	0.2590	0.1	0.3250
Precipitation × Warming	6	64	0.4	0.8851	0.7	0.6603
Precipitation × Winter rain	3	64	0.2	0.9127	0.4	0.7453
Precipitation × Warming × Flowering	6	192	1.1	0.4099	0.9	0.5221
Precipitation × Winter rain × Flowering	3	192	0.7	0.5647	1.4	0.2462
Warming × Year	2	192	0.0	0.9608	0.3	0.7210
Precipitation × Year	3	192	1.4	0.2494	0.6	0.5267
Winter rain × Year	1	192	0.7	0.4209	0.1	0.7719
Flowering × Year	1	192	1.2	0.2763	2.4	0.1257
Warming × Flowering × Year	2	192	0.4	0.6674	0.6	0.5473
Precipitation × Flowering × Year	3	192	0.3	0.8306	0.6	0.5852
Winter rain × Flowering × Year	1	192	1.0	0.3284	0.4	0.5405
Precipitation × Warming × Year	6	192	0.1	0.9994	0.1	0.9965
Precipitation × Winter rain × Year	3	192	0.2	0.8738	0.3	0.8162
Precipitation × Warming × Flowering × Year	6	192	1.5	0.1960	1.5	0.1970
Precipitation × Winter rain × Flowering × Year	3	192	0.1	0.9345	0.3	0.7914

6.2 Manuscript 2

Title: Climatic extremes lead to species-specific legume facilitation in an experimental temperate grassland

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Keywords: Biomass production; ^{15}N natural abundance; Mesic; Plant-plant interactions; Precipitation; Species-specific traits; Water stress.

Abstract

Background Nitrogen-fixing legumes are key species in grassland ecosystems, as their ability to fix atmospheric nitrogen can facilitate neighboring plants. However, little is known about the fate of this legume effect in the face of extreme weather events, which are increasingly expected to occur.

Methods Here, we examined experimentally how the presence of a legume modifies above-ground net primary production (ANPP) and nitrogen supply of neighboring non-legumes under annually recurrent pulsed drought and heavy rainfall events by comparing responses of three key species in European grassland versus without legume presence over 4 years.

Results Legume presence facilitated community productivity of neighboring non-legumes under ambient weather conditions and also under experimental heavy rainfall. However, no facilitation of community productivity by the legume was found under experimental drought. Productivity of the three target species responded species-specifically to legume presence under different weather conditions: *Holcus lanatus* was facilitated only under control conditions, *Plantago lanceolata* was facilitated only under heavy rainfall, and *Arrhenatherum elatius* was facilitated irrespective of climate manipulations. The legume effects on δ^{15}N, leaf N concentration, and N uptake were also species-specific, yet irrespective of the climate manipulations. The data suggest that the missing legume effect on community productivity under the pulsed drought was rather caused by reduced N-uptake of the target species than by reduced N-fixation by the legume.

Conclusions In contrast to heavy rain, the presence of a legume could not effectively buffer community ANPP against the negative effects of extreme drought events in an experimental temperate grassland. Facilitation also depends on the key species that are dominating a grassland community.

Abbreviations and terms

ANPP	Above-ground net primary production
Arr	*Arrhenatherum elatius* (L.) P. Beauv. ex J. Presl & C. Presl
Ger	*Geranium pratense* L.
Hol	*Holcus lanatus* L.
Leg	Legume
Lot	*Lotus corniculatus* L.
Legume neighbors-	A single mixture of three target non-legumes grown in the presence of a legume forb (*L. corniculatus*)
Non-legume neighbors-	A single mixture of three target non-legumes grown in the presence of a non-legume forb (*G. pratense*)
N	Nitrogen
Pla	*Plantago lanceolata* L.
Clim	Climate manipulations

Introduction

The presence of nitrogen fixing legumes has positive effects on productivity in Central European grassland ecosystems (Temperton et al. 2007; Marquard et al. 2009; Küchenmeister et al. 2012; Roscher et al. 2012). Evidence suggests that the effects of declining species richness on ecosystem functioning can partly be buffered by the presence of key species that control the functioning of communities (Beierkuhnlein and Jentsch 2005; Hooper et al. 2005). Legumes are such key species that are crucial for ecosystem productivity and stability (Spehn et al. 2002; Temperton et al. 2007; Marquard et al. 2009). However, there is a substantial lack of experimental evidence regarding facilitative legume effects on ANPP of neighboring grassland species under extreme weather conditions.

Extreme weather events such as severe drought and heavy rainfall are likely to have strong effects on vegetation and ecosystem functions like ANPP (Stampfli and Zeiter 2004; Grime et al. 2008;

Beierkuhnlein et al. 2011; Dreesen et al. 2012). Such extreme conditions are expected to increase in frequency and magnitude as a consequence of global climate change (Easterling et al. 2000; Jentsch and Beierkuhnlein 2008; IPCC 2012; Rummukainen 2012). Experimental approaches to scrutinize whether impacts of climate extremes can be modified by plant species richness and community composition have been applied in various grassland ecosystems (e.g. Jentsch et al. 2007; Fay et al. 2008; Smith 2011). However, as yet the potential contribution of legume species to the buffering of ANPP reduction in the face of recurrent, elongated drought or heavy rainfall has not been widely investigated.

Under ambient weather conditions, generally all legumes have the ability to fix atmospheric nitrogen via their symbiotic microbial partners (Mosier 2002). Therefore, legumes often do not compete with neighboring plants for soil nitrogen (Paynel et al. 2001), and a surplus of fixed nitrogen can become available when legume plant parts decompose and are mineralized (Tomm et al. 1995). N_2 fixed by legumes can also be transferred to neighboring plants via root exudation (Paynel et al. 2001; Pirhofer-Walzl et al. 2012). Neighboring plants growing close to legumes can benefit from additional nutrient supply (Chu et al. 2004). As a consequence, shoot N uptake, leaf N concentration, $\delta^{15}N$ status, and ANPP of neighboring plants are modified by legume presence (Temperton et al. 2007).

Nitrogen fixation and productivity of legumes can be greatly modified at any time during the growing period when soil moisture is inadequate (Striker et al. 2005; Pimratch et al. 2008; Silvente et al. 2012). Symbiotic N_2 fixation by legumes is highly sensitive to water stress or drought as nitrogenase activity depends on the amount of phloem flow into the nodules, which decreases under water stress (Serraj et al. 1999; Marino et al. 2007). Water stress inhibits all aspects of N_2 fixation including nodulation, nodule development, and nodule activity (Serraj et al. 1999; Aranjuelo et al. 2007).

On the other hand, soil O_2 concentration is particularly critical to the N_2 fixing function of legumes. An adequate amount of O_2 is required by the rhizobial-infected cells to provide energy for N_2 fixation (Witty et al. 1986). Therefore, any reduction in O_2 supply to the nodules under waterlogging condition leads to poor performance of nodulated legumes (Pugh et al. 1995; James and Crawford 1998). In temperate regions some legumes of economic importance such as *Lotus corniculatus* have adapted to grow, nodulate, and fix atmospheric N_2 even under waterlogged conditions (Pugh et al. 1995; James and Crawford 1998; Striker et al. 2005). However, it is necessary to know how legume species perform under annually recurrent pulsed drought and heavy rainfall, which in turn influences their facilitative potential on ANPP of neighboring plant species.

In addition, symbiotic N_2 fixation by legumes and above ground biomass production of experimental grassland is reported to steadily increase over at least the first four years after community establishment (Mulder et al. 2002; Hooper and Dukes 2004; Roscher et al. 2011). That is why legume facilitation effects on ANPP of neighboring non-legumes may become stronger in the years after community establishment, in particular on N-limited sites. Thus, it is also important to observe how legume facilitation modifies ANPP over time.

We compared the responses of legume neighbors and non-legume neighbors in a long term precipitation manipulation experiment during 2007-2010 (EVENT I experiment, Jentsch et al. 2007). We hypothesized that (1) legume neighbors would have significantly higher ANPP, higher δ^{15}N (which is closer to zero), higher leaf N concentration and higher shoot N uptake compared to non-legume neighbors, and that (2) the effects of legume facilitation on ANPP of neighboring non-legumes increases over time. Further, we expected that (3) under annually recurrent pulsed drought, legume neighbors would not have significantly higher ANPP, higher δ^{15}N (which is closer to zero), higher leaf N concentration, and higher shoot N uptake compared to non-legume neighbors. We also hypothesize that (4) under annually recurrent heavy rainfall, legume neighbors would not have significantly higher ANPP, higher δ^{15}N (which is closer to zero), higher leaf N concentration, and higher shoot N uptake compared to non-legume neighbors.

Materials and methods

Experimental site

The experiment was set up in the Ecological Botanical Garden of the University of Bayreuth, Germany (49˚55′19″N, 11˚34′55″E, 365 m asl) in the year 2005 and has operated since (Jentsch et al. 2007, 2011). Mean annual temperature of the site is 8.2 ˚C and mean annual precipitation is 724 mm (1971-2000). Precipitation is distributed bi-modally with a major peak in June-July and a second peak in December-January (data: German Weather Service). The experimental site was built up artificially (about 80 cm in depth) with homogenized sandy subsoil from a nearby quarry. Underlain drainage facilities were installed to avoid soil related heterogeneity. The upper layer (20 cm depth) consisted of homogenized topsoil of the quarry containing higher amounts of organic material. The texture of the soil body is loamy sand (82% sand, 13% silt, 5% clay) with $pH_{(KCl)}$ = 4.5 and total N = 0.07% in the upper layer, and with $pH_{(KCl)}$ = 6.2 and total N = 0.01% in the lower soil layer.

Experimental design

The EVENT I -experiment has been set up with two fully crossed factors: (i) legume presence versus absence (pair wise comparison of a single mixture of three grassland non-legumes with one legume forb versus the same mixture of three non-legumes with another non-legume forb) and (ii) climate manipulations (control, drought and heavy rainfall). The experimental design consisted of 30 plots, each 2×2 m in size and five replicates of each factorial combination. However, data acquisition was carried out in the central square meter of each plot only in order to avoid edge effects. Plots were blocked pair wise (legume presence versus absence) and randomly assigned within each of the five replications of climate manipulations (control, drought, heavy rainfall). One hundred plant individuals per plot were planted in defined quantitative composition in a systematic hexagonal grid with 20 cm distance between individuals in April 2005. We maintained the composition of originally installed species by regular weeding. Weeds were removed as juveniles and did not produce significant amounts of biomass (and therefore also are considered negligible with regards to competition).

Experimental grassland communities

We investigated two grassland communities, a legume community and a non-legume community, both being composed of four species. We planted three target non-legumes, the two common European grasses *Arrhenatherum elatius* (L.) P. Beauv. ex J. Presl & C. Presl, *Holcus lanatus* L. and the forb *Plantago lanceolata* L. either together with a legume forb (*Lotus corniculatus* L.), or with a non-legume forb (*Geranium pratense* L.). This substitutive design results in same species number, (initial) density, and (initial) abundances per species of the two communities. *L. corniculatus and G. pratense* are perennial, generative and develop similar root systems with persistent taproots that survive over their entire life-span (Klimešová and de Bello 2009; Kutschera et al. 2009). The genet life-span of both species varies between 15-30 years. Based on this, we assumed that the legume and its replacement would also be comparable in their productivity and morphology, and thus their competitiveness. However, the legume *L. corniculatus* turned out to be much more productive than its replacement *G. pratense* (see results). Consequently, the competitive effect of the legume is much higher than that of the forb. Any increased performance (productivity, N-status) of the neighboring target species in the presence of the productive legume as compared to the presence of the unproductive forb can therefore be interpreted as a (strong) indication for a facilitating legume effect.

All species used for this experiment were selected with respect to their affiliation to functional groups (grasses, forbs, legume forbs), to life span (perennials), and to their overall importance in nearby and Central European managed grassland ecosystems. Plants used in the experiment were grown from seeds in a greenhouse and even aged when being planted. All plants were acclimated on site since February 2005, reaching heights of approximately 15 cm. Biomass at planting amounted to 0.1–0.6 g dry weight per individual. Data collection for this study started in 2007.

Climate manipulations

The climate treatment involved either annually recurrent pulsed drought or heavy rainfall events in early summer and ambient conditions for control. Intensity of the climate manipulations was based on the local 100-year extreme event in 2007, and then was intensified to the local 1000-year extreme event during the years 2008-2010. We determined extremeness of weather treatments by statistical extremity with respect to a historical reference period (extreme value theory), independent of its effects on vegetation (Jentsch 2006). We used the growing seasons (March–September) of 1961–2000 as a reference period (data: German Weather Service). Gumbel I distributions were fitted to the annual extremes, and 100 or 1000-year recurrent events were calculated. We defined drought as the number of consecutive days without effective precipitation (<1 mm/day) during the growing season.

The control plots remained without climate manipulation throughout the entire period. We maintained drought plots under rain-out shelters during climate manipulations. These were constructed with a steel frame (Hochtunnel, E & R Stolte GmbH, Germany), and covered with a transparent plastic sheet (material: 0.2 mm polyethylene, SPR 5, Hermann Meyer KG, Germany). Rain-out shelters permitted nearly 90% penetration of photosynthetically active radiation. A total of 32 days of drought in 2007 and of 42 days of drought in 2008-2010 was applied in the experiment during the peak growing season in June. Maximum values in the historical data set were 33 days without rain during June and July 1976. We removed the roofs after the experimental drought period. Greenhouse effects due to rain-out shelters were minimized by having an 80 cm clearance between the roof and the ground, allowing for near-surface air exchange. Near-surface air temperature was increased by 1.2 °C during the climate manipulation period. Natural drought periods are usually also accompanied by heat (De Boeck et al. 2010), implying that warming is a realistic scenario for drought periods. We further used artefact controls (irrigating the amount of natural rainfall under intact rain-out shelters) and did not find significant roof artifacts (Kreyling et al. 2008).

We applied heavy rainfall using portable irrigation systems by Veejet 80100 nozzles. Drop size and rainfall intensity resembled natural heavy rainfall events. We manipulated local 100 or 1000-year heavy rainfall extremes according to extreme value statistics, i.e. 170 mm precipitation during 14 days or 260 mm during 21 days, respectively. Maximum values in the historical data set were 152 mm precipitation during 14 days in June 1977. The daily amount of water (around 12.5 mm) was applied in two applications to ensure high soil water saturation. The amount of water added through natural precipitation (if any) was balanced by subtracting the amount of rain from the respective dose. Lateral surface flow was avoided in all plots (control, drought and heavy rainfall) by barriers of plastic sheet pilings around treated plots reaching down to a depth of 10 cm.

Soil moisture in the main rooting zone (between 2-7 cm depth) was recorded by FD-sensors (Echo.EC-5 ⁄ k; Decagon Devices, Pullman, WA, USA). Data were taken from one grassland plot per treatment block in 1-hour intervals. Soil moisture content significantly varied between climate manipulations (control, drought and heavy rain; Fig. 1, $F_{(2, 97)} = 102.5$, $p < 0.0001$). Soil moisture content was lowest during drought and highest during heavy rainfall manipulations. Control plots received only natural precipitation, thus soil moisture content strongly differed between the drought and the heavy rain plots. The year 2008 was extremely dry making the heavy rain manipulation more effective in terms of water supply. Nonetheless, soil moisture values occurred below the wilting point (pF = 4.2) for longer periods at the drought plots during climate manipulations in all years.

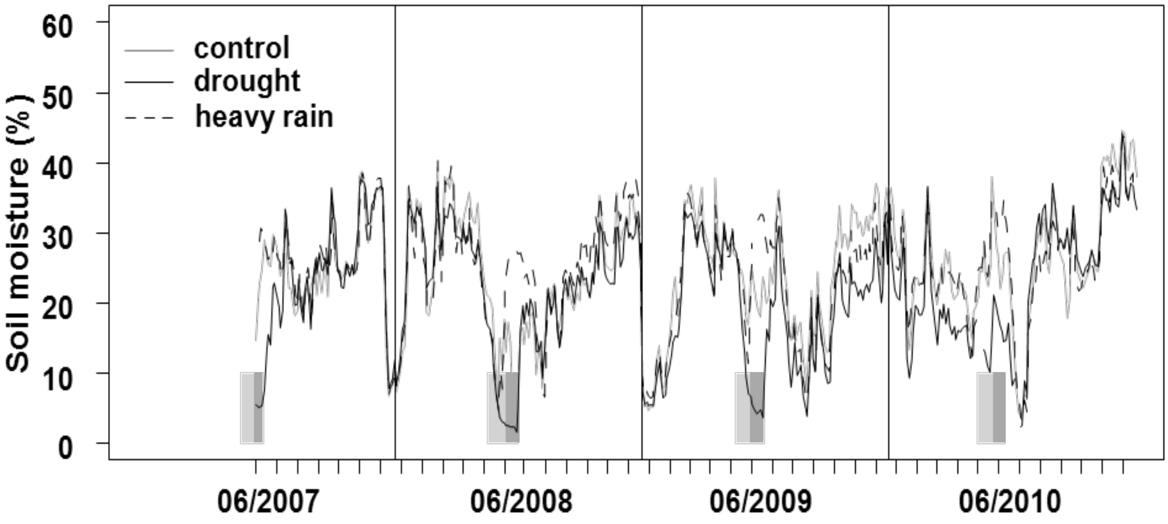


Fig. 1 Soil moisture (%) under three experimental climatic conditions over time. Soil moisture content was lowest during drought (light and dark grey boxes) and highest during heavy rainfall (dark grey boxes only) manipulations. Soil moisture content significantly varied among treatments ($F_{(2, 97)} = 102.5$, $p < 0.0001$).

Response parameters

Above-ground biomass production

Standing plant biomass of all target species was harvested twice a year (at the end of climate manipulations in early July and again in mid-September), resembling local agricultural routines. Collected samples were sorted to species and dried to a constant weight at 75 ˚C and weighed (Ohaus NavigatorTM, Ohaus Corporation, Parsippany, NJ, USA; accuracy ± 0.01 g). ANPP was calculated as the sum of both harvests per year. Total ANPP (gm^{-2} $year^{-1}$) of the three target species *A. elatius, H. lanatus, and P. lanceolata* were used for analysis. Biomass data were further used for measuring response ratio, which signified the effect size of legume facilitation (presence and absence) on ANPP of the neighboring target species. The effect size of legume facilitation was calculated by: ln (*Legume effect size)* = ln (X_{+Leg} / X_{-Leg}), where X_{+Leg} represents the ANPP of the target species in presence of the legume and X_{-Leg} represents the ANPP of the target species in absence of a legume.

Plant nitrogen status

Evidence for legume facilitation related to N_2 fixation was measured by using the δ^{15}N technique (Högberg 1997; Temperton et al. 2007). The transfer of N_2 from legume species to their neighbors can be tracked via an isotopic signature of δ^{15}N that is closer to atmospheric δ^{15}N than N taken up from the soil. Thus, evidence regarding N_2 transfer from legumes to their neighbors can be presented if δ^{15}N values of legume neighbors are closer to zero compared to non-legume neighbors. Högberg (1997) and Jumpponen et al. (2002) argue that irrespective of legume effect, other mechanisms like δ^{15}N signal of soil organic matter, species-specific ability of N uptake and plant mixture within the community can also modify the δ^{15}N status. Here, we used three target species in two plant communities, a legume community and a non-legume community, both having four species. The only difference between the two plant communities was the presence and absence of the legume forb. We replaced the legume forb (*L. corniculatus*) by a non-legume forb (*G. pratense*) and assumed that the altered δ^{15}N values for the neighboring plants are due to the legume effect rather than other mechanisms. However, the differences in $^{15}N/^{14}N$ ratios between plant-available soil N and N coming from N_2 fixation were small in our study. Therefore, together with δ^{15}N signals, we also measured legume δ^{15}N, shoot N content and leaf N concentration, as suggested by Högberg (1997), which provides a good way to assess the facilitative effect of legume presence on their neighbors under the three climate treatments.

Equally aged south-facing leaves of the target species were collected to determine N-isotope ratios and total leaf N concentrations in July 2007. Leaves were oven dried at 60 ˚C for 48 h and then fine-milled for analysis. Samples (2.25 ± 0.1 mg) were analyzed using an elemental analyzer (EA 3000; Euro Vector, Italy) coupled online to a ConFlo III interface connected to an isotope-ratio mass spectrometer (MAT 253; Thermo Electron). Leaf N concentrations (%) per species per plot were provided by this analysis. Ratio of $^{15}N:^{14}N$ was also reported as the international standard for atmospheric nitrogen. Then δ^{15}N values were calculated as: $\delta^{15}N$ [‰] = (Rsample / Rstandard) – 1×10^3, where R represents the ratio of $^{15}N:^{14}N$ isotopes. Shoot N uptake or shoot N content (gm^{-2}) was calculated by multiplying the total above ground dry biomass by the respective leaf N concentration (%). Here, shoot N based on leaf N concentration and total aboveground biomass might result in somewhat wrong absolute values as N concentration varied among plant parts. Leaves always have the highest N concentration, about 4.0-4.6 % of dry matter content (DM) compared to shoot, stubble and root in the range 2.5-3.5 %, 2-3 % and 2-2.7 % of DM, respectively (Huss-Danell and Chaia 2005). However, Huss-Danell and Chaia (2005) also suggested that leaves can provide useful indications of N content in shoots and whole plants. Yet we are interested in the relative differences between the treatments in our study and assume that the ratio of N concentration in different plant organs does not differ between treatments.

Soil nitrogen status

Soil nitrogen status was obtained from four homogenized, sieved (< 2 mm), mixed samples of the upper soil layer (between 0-10 cm depth) of each plot sampled in July 2008. Plant-available soil nitrogen was extracted using a 1 M KCl solution. After filtration (Typ 15 A Blauband; Roth, Karlsruhe, Germany), nitrate and ammonium concentrations were quantified using flow injection analysis (measurements conducted at BayCEER Analytical Chemistry, Bayreuth, device: FIA-LAB, MLE GmbH, Dresden, Germany).

Statistical analysis

Linear Mixed-Effects Models combined with analysis of variance (ANOVA) were used to test for significant differences between groups at single points of time while taking the split-plot design into account by using block identity as a random factor (e.g., for species-wise analysis of δ^{15}N, shoot N uptake, leaf N concentration and plot wise analysis of soil N status). Species was set as an additional random factor before performing an overall analysis of δ^{15}N and leaf N concentration. For total shoot N uptake analysis, we summed up the values of our three target grassland species. The fixed factors were legume presence and climate manipulations, which were also tested for their interaction. For time series analyses, Linear Mixed-Effects Models combined with ANOVA were applied to test for the interaction of legume presence and climate manipulations while taking the split-plot design and the repeated measures into account (block identity and time used as random factors). In this case, total ANPP of the three target species were used as response parameter. Homogeneous groups of factor combinations (legume presence, climate manipulations) were identified by Tukey’s HSD post hoc comparisons. Prior to statistical analysis, data were ranked (only for shoot N uptake) or square-root or log transformed to improve the homogeneity of variances, or if conditions of normality were not met. All characteristics were tested by examining the residuals versus fitted plots and the normal qq-plots of the linear models (Faraway 2005). Level of significance was set to $p < 0.05$. Linear Mixed-Effects Models were conducted with the function ‘lme’ (package nlme, Pinheiro et al. 2012) and graphs were created using the package sciplot (Morales and R-Development-Core-Team 2012) in R version 2.13.2 (The R Project for Statistical Computing, R-Development-Core-Team 2011).

Results

Effects of legume presence irrespective of climate manipulations

Experimental grassland species growing as legume neighbors had significantly higher total ANPP compared to those growing as non-legume neighbors (Total, $F_{(1, 95)} = 63.8$, $p < 0.0001$; Fig. 2a and 3). Legume neighbors had significantly higher δ^{15}N values which were closer to zero ($F_{(1, 68)} = 4.5$, $p = 0.0383$; Table 1), higher leaf N concentration ($F_{(1, 70)} = 15.2$, $p = 0.0002$; Table 1) and higher shoot N uptake ($F_{(1, 20)} = 13.4$, $p = 0.0016$; Fig. 4) compared to non-legume neighbors. The legume effects on δ^{15}N, leaf N concentration, and shoot N uptake were species-specific. δ^{15}N values of *P. lanceolata* ($F_{(1,20)} = 5.5$, $p = 0.0292$), leaf N concentration of *A. elatius and P. lanceolata* (Arr- $F_{(1,20)} = 10.8$, $p = 0.0037$; Pla- $F_{(1,20)} = 6.3$, $p = 0.0211$), and shoot N uptake of *A. elatius and H. lanatus* (Arr- $F_{(1,20)} = 13.1$, $p = 0.0.0017$, Hol- $F_{(1,20)} = 8.0$, $p = 0.0105$) were significantly promoted by legume presence. Soil N status was higher in the presence of legumes (NH_4^+: $F_{(1, 19)} = 4.9$, $p = 0.0399$; NO_3^-: $F_{(1, 19)} = 1.3$, $p = 0.2670$; N%: $F_{(1, 19)} = 4.6$, $p = 0.0443$; Fig. 5).

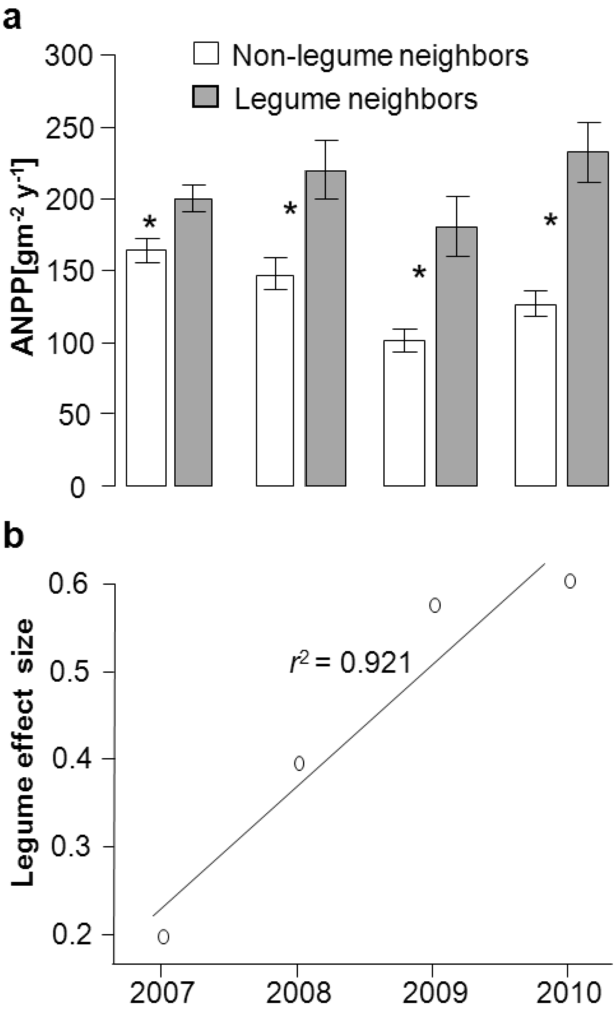
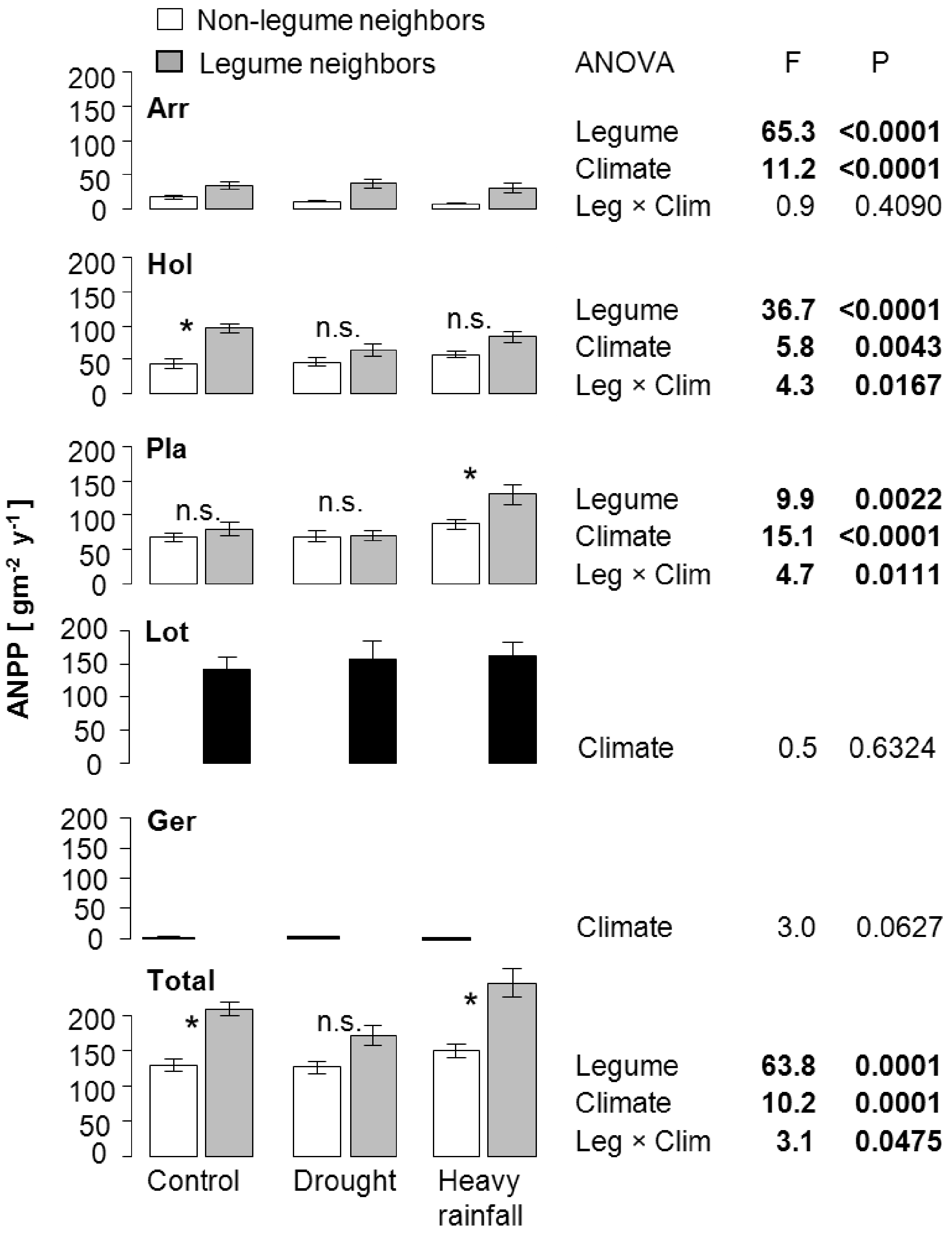
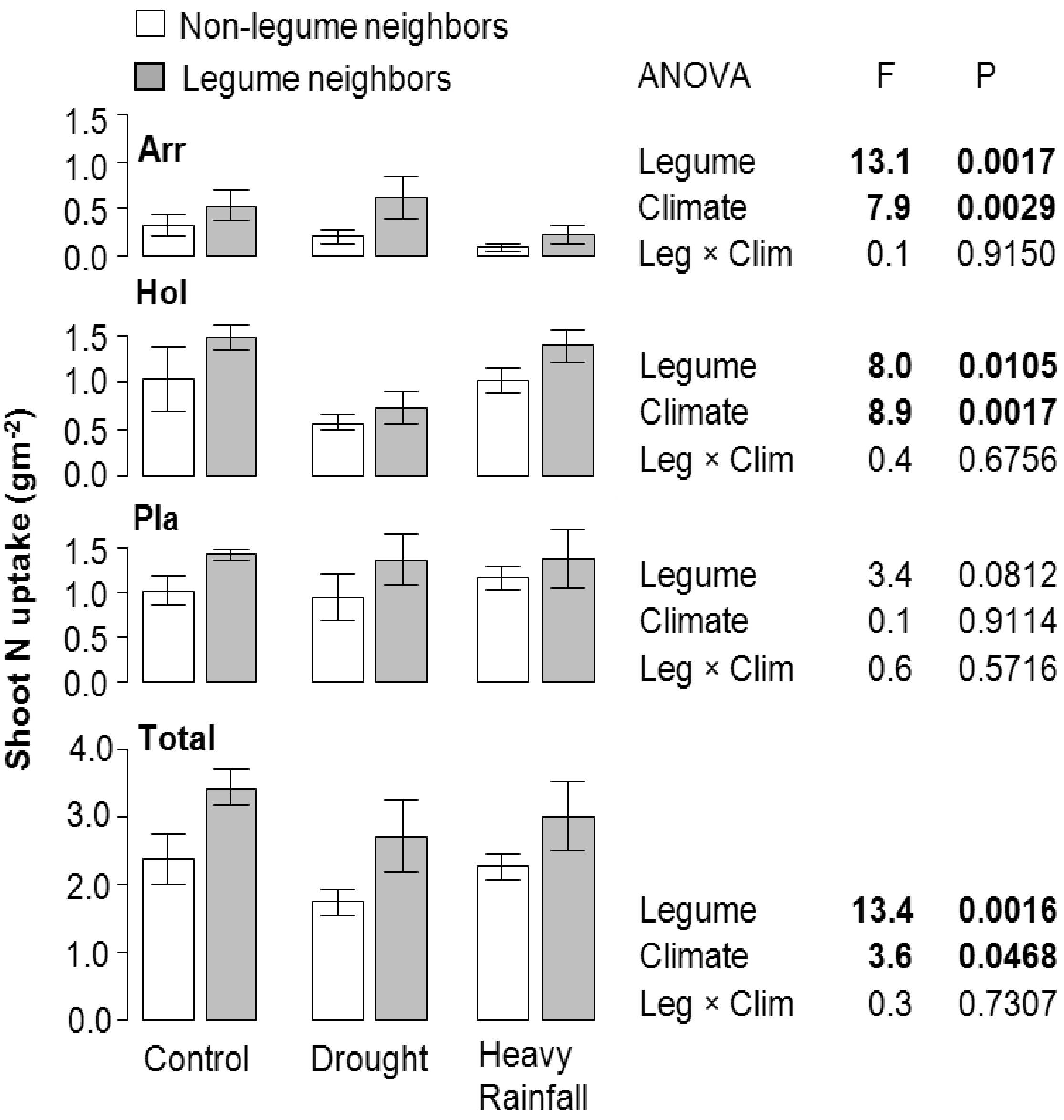


Fig. 2 Irrespective of climate manipulations, (a) effect of presence and absence of legume on ANPP and (b) legume effect size over time. Mean values and standard errors of 15 replications are shown. Significant pair wise variation in ANPP due to the presence and absence of legume are marked with asterisks, $p < 0.05$. See method for details about legume effect size.



ANOVA	F	P
Legume	**65.3**	**<0.0001**
Climate	**11.2**	**<0.0001**
Leg × Clim	0.9	0.4090
Legume	**36.7**	**<0.0001**
Climate	**5.8**	**0.0043**
Leg × Clim	**4.3**	**0.0167**
Legume	**9.9**	**0.0022**
Climate	**15.1**	**<0.0001**
Leg × Clim	**4.7**	**0.0111**
Climate	0.5	0.6324
Climate	3.0	0.0627
Legume	**63.8**	**0.0001**
Climate	**10.2**	**0.0001**
Leg × Clim	**3.1**	**0.0475**

Fig. 3 Legume effect on ANPP under three experimental climatic conditions (control, drought and heavy rainfall). Mean values and standard errors of five replications over 4 years (2007-2010) are shown as the sum of two harvests per year. Significant variations are marked in bold ($p < 0.05$) and significant pair wise comparisons are indicated by asterisk (Tukey's post-hoc, $p < 0.05$) in case of significant interactions between legume presence and climate manipulations. ANPP of three target species (Arr, Hol, and Pla) were summed up (Total).

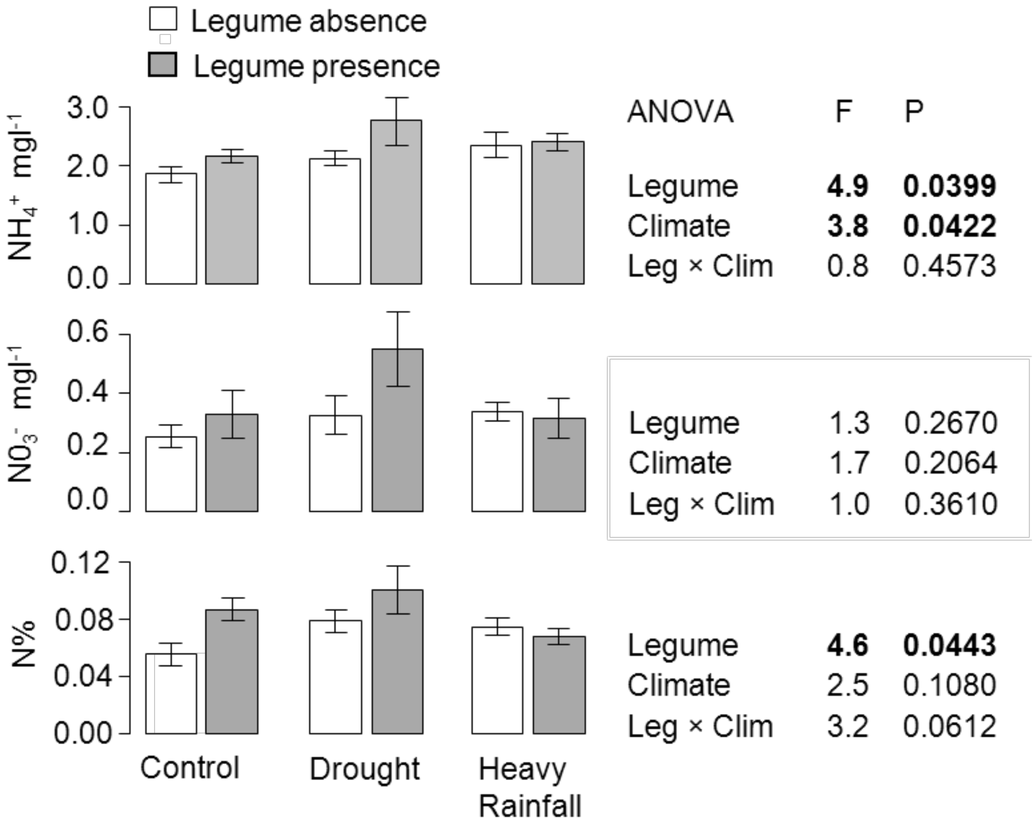


ANOVA	F	P
Legume	**13.1**	**0.0017**
Climate	**7.9**	**0.0029**
Leg × Clim	0.1	0.9150
Legume	**8.0**	**0.0105**
Climate	**8.9**	**0.0017**
Leg × Clim	0.4	0.6756
Legume	3.4	0.0812
Climate	0.1	0.9114
Leg × Clim	0.6	0.5716
Legume	**13.4**	**0.0016**
Climate	**3.6**	**0.0468**
Leg × Clim	0.3	0.7307

Fig. 4 Effect of legume presence and absence on N uptake under three climate conditions (data from 2007). Significant variations are marked in bold ($p < 0.05$). Legume presence and climate manipulations had significant effect on total N uptake. N uptakes of three target species (Arr, Hol and Pla) were summed up (Total).

Patterns of legume facilitation effect on ANPP over time

Irrespective of climate manipulations, legume facilitation on ANPP gradually increased over the whole experimental period, 2007-2010 (Linear regression, slope = 0.14, r^2 = 0.921; Fig. 2b). This increasing legume facilitation effect was strongest in control (Linear regression: slope = 0.18, r^2 = 0.996) and in the heavy rainfall manipulation (slope = 0.16, r^2 = 0.736) and effect sizes were smaller for the drought manipulation (slope = 0.07, r^2 = 1.000; Fig. 6).



ANOVA	F	P
Legume	**4.9**	**0.0399**
Climate	**3.8**	**0.0422**
Leg × Clim	0.8	0.4573

Legume	1.3	0.2670
Climate	1.7	0.2064
Leg × Clim	1.0	0.3610

Legume	**4.6**	**0.0443**
Climate	2.5	0.1080
Leg × Clim	3.2	0.0612

Fig. 5 Effect of presence and absence of legume on soil N status (NH_4^+, NO_3^- and N %) under three climate conditions (data from 2008). Significant results ($p < 0.05$) are marked in bold.

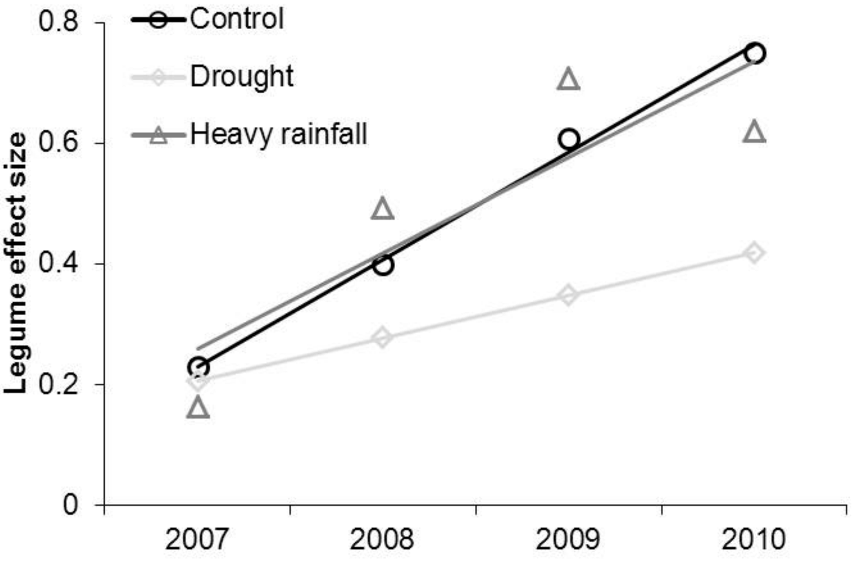


Fig. 6 Legume effect size under control, drought and heavy rainfall gradually increases over time. Linear regression: control (black line), slope = 0.18, r^2 = 0.996; drought (light grey line), slope = 0.07, r^2 = 1.000; and heavy rainfall (dark grey line), slope = 0.16, r^2 = 0.736 (See method for details about legume effect size).

Table 1 Effect of presence (+) and absence (-) of legume on δ^{15}N and leaf N concentration under three climate conditions (control, drought and heavy rainfall). Mean values and standard deviations for five replications are shown here. Significant (ANOVA, $p < 0.05$) results are set bold.

Response Parameters	Species	Control		Drought		Heavy rainfall		ANOVA		
		-Legume	+Legume	-Legume	+Legume	-Legume	+Legume	Leg $P(F)$	Clim $P(F)$	Leg × Clim $P(F)$
δ^{15}N (‰)	Arr	-2.07 ± 0.1	-1.70 ± 0.76	-2.30 ± 0.97	-1.75 ± 0.53	-2.23 ± 1.88	-2.11 ± 0.95	0.2367(1.5)	0.7121(0.3)	0.8287(0.2)
	Hol	-2.69 ± 1.28	-1.82 ± 0.56	-2.52 ± 1.42	-2.58 ± 1.40	-1.24 ± 1.73	-2.03 ± 1.20	0.7953(0.1)	0.0966(2.7)	0.3012(1.3)
	Pla	-3.65 ± 1.20	-1.55 ± 1.10	-2.49 ± 1.70	-1.72 ± 1.08	-3.47 ± 1.87	-2.84 ± 1.29	**0.0292(5.5)**	0.2476(1.5)	0.4253(0.9)
	Overall[a]	-2.81 ± 1.27	-1.69 ± 0.78	-2.43 ± 1.29	-2.02 ± 1.07	-2.31 ± 1.94	-2.33 ± 1.13	**0.0383(4.5)**	0.9182(0.1)	0.1839(1.7)
Leaf N concentration (%)	Arr	1.87 ± 0.26	2.16 ± 0.27	1.47 ± 0.14	1.97 ± 0.44	1.62 ± 0.34	2.22 ± 0.66	**0.0037(10.8)**	0.2414(1.5)	0.6555(0.4)
	Hol	1.68 ± 0.21	1.76 ± 0.06	1.18 ± 0.07	1.29 ± 0.14	1.81 ± 0.27	1.94 ± 0.29	0.1411(2.3)	**<0.0001(29.7)**	0.9676(0.0)
	Pla	1.10 ± 0.21	1.40 ± 0.24	1.09 ± 0.23	1.39 ± 0.35	1.04 ± 0.16	1.11 ± 0.35	**0.0211(6.3)**	0.2227(1.6)	0.5187(0.7)
	Overall[a]	1.55 ± 0.40	1.77 ± 0.38	1.25 ± 0.22	1.55 ± 0.44	1.49 ± 0.42	1.76 ± 0.65	**0.0002(15.2)**	**0.0050(5.7)**	0.8947(0.1)

Note: [a] block and species were used as random factor for overall analysis.

Effects of legume presence under drought

Under annually recurrent drought conditions, total ANPP of the three target species was not affected by legume presence (significant interaction between climate and legume treatment; post-hoc test of the legume effect for drought only: p = 0.1246; Fig. 3). Similarly, the effects of legume presence on ANPP were not significant for *H. lanatus*, and *P. lanceolata* (again significant interaction between climate and legume treatment; post-hoc test of the legume effect for drought only: Hol, p = 0.3243; Pla, p = 0.9999; Fig. 3). The overall legume effects were not modified by the interaction of climate manipulations for *A. elatius*, implying that this target species was facilitated by legume presence also under drought (interaction between climate and legume treatment, p = 0.4090; Fig. 3). Irrespective of the legume effect, total ANPP of all target species was not significantly affected by the drought manipulation.

The effects of legume presence on δ^{15}N, leaf N concentration, and shoot N uptake over all species were not altered by the interaction between climate and legume treatments and therefore persisted also under drought conditions (Table 1, Fig. 4). But, both legume and non-legume neighbors grown under drought conditions showed a tendency for lower leaf N concentrations compared to those plants grown under control conditions (Climate, $F_{(2,70)}$ = 5.7, p = 0.0050, Table 1; Control versus Drought, p = 0.0572). Shoot N uptake significantly varied between climate manipulations ($F_{(2,20)}$ = 3.6, p = 0.0468) and it was slightly lower under drought compared to control (Fig. 4). At the species level, leaf N concentration and shoot N uptake of *H. lanatus* were significantly lower under drought conditions compared to control (Control versus drought: Leaf N concentration, $p < 0.0001$, Shoot N uptake, p = 0.0053; Legume neighbors under control versus legume neighbors under drought: Leaf N concentration, p = 0.0098, Shoot N uptake, p = 0.0536). The significant legume effects on δ^{15}N in *P. lanceolata* and leaf N concentration in *A. elatius* and *P. lanceolata* were not affected by the climate manipulations (Table 1). Likewise, the legume effect on N-uptake of *A. elatius* and *H. lanatus* also persisted under drought conditions (Fig. 4).

Among the three response parameters of soil N status (NH_4^+, NO_3^- and N %) only soil NH_4^+ significantly varied between climate manipulations (Climate, $F_{(2, 19)}$=3.8, p=0.0422). Soil NH_4^+ and soil N% furthermore showed positive legume effects irrespective of climate manipulations (Fig. 5).

Effects of legume presence under heavy rainfall

Total ANPP of the three target species was positively affected by legume presence under heavy rainfall conditions (significant interaction between climate and legume treatment; post-hoc test of the legume effect for heavy rainfall only: $p < 0.0001$; Fig. 3). This pattern was mainly driven by *P.*

lanceolata, which showed a significant positive legume effect only under heavy rainfall (post-hoc test of the legume effect for heavy rainfall: $p = 0.0024$, Fig. 3). ANPP of *H. lanatus* was not facilitated under heavy rainfall, while ANPP of *A. elatius* was persistently facilitated by legume presence also under heavy rainfall (significant main effect but no significant interaction with climate manipulations, Fig. 3). In addition, irrespective of legume effects, heavy rainfall significantly increased total ANPP of the target species by 18% compared to control ($p = 0.0317$). Leaf N concentration was also significantly impacted by the climate manipulations ($F_{(2,70)} = 5.7$, $p = 0.0050$, Table 1), and under heavy rainfall it was slightly higher in legume neighbors (1.76 ± 0.65) compared to non-legume neighbors (1.49 ± 0.42). At the species level, leaf N concentration of *H. lanatus* was significantly higher under heavy rainfall compared to drought (Heavy rainfall versus Drought, $p < 0.0001$; Legume neighbor under heavy rain versus legume neighbor under drought, $p = 0.0002$). The significant legume effects of overall δ^{15}N, total shoot N uptake, individual N-uptake of *A. elatius* and *H. lanatus*, and soil NH_4^+ persisted under the heavy rainfall manipulation (Table 1 and Fig. 4, 5). In addition, heavy rainfall had no significant effect on soil N status (Fig. 5).

Discussion

Effects of legume presence irrespective of climate manipulations

We confirmed the first hypothesis that target species will profit from legume presence as indicated by increased ANPP, higher δ^{15}N, higher leaf N concentration, and higher shoot N uptake compared to non-legume neighbors (Table 1, Fig. 2a, Fig. 3, and Fig. 4). Concerning isotopic signature of δ^{15}N, leaf N concentration, and shoot N uptake, responses of the three target species to legume presence were found to be species-specific (Table 1 and Fig. 4). δ^{15}N values of *P. lanceolata* significantly depended on legume presence but the δ^{15}N values of the other two target species, *A. elatius* and *H. lanatus*, were not significantly modified by legume presence. Along with *P. lanceolata*, leaf N concentration of *A. elatius* benefited from legume presence. Furthermore, legume presence had a significant effect on shoot N uptake of *A. elatius* and *H. lanatus*, yet shoot N uptake of *P. lanceolata* did not significantly vary with legume presence. Soil N status data confirmed that the legume presence had beneficial effects on soil NH_4^+ and N% (Fig. 5).

Comparable positive effects of legume presence on nutrient availability, uptake, and growth of neighboring species are reported from many other studies (e.g. Tomm et al. 1995; Høgh-Jensen and Schjoerring 2000; Paynel et al. 2001; Spehn et al. 2002; Chu et al. 2004; Temperton et al. 2007; Marquard et al. 2009; von Felten et al. 2009; Küchenmeister et al. 2012; Pirhofer-Walzl et al. 2012; Roscher et al. 2012). This underlines that legumes play a crucial role for the performance of grasslands by supporting the fixation of atmospheric N_2 which becomes available for the whole plant community. Apparently, legume presence also reduces the competition for soil N (“nitrogen sparing”) as legumes rely more on atmospheric N_2 than on soil N. However, our study provides novel insights by demonstrating species-specific responses to legume presence, i.e. species-specific facilitation.

We used a substitutive design where the legume *L. corniculatus* was replaced by a non-legume forb, *G. pratense*. Despite *L. corniculatus* being much more productive than *G. pratense* (Fig. 3), and thus probably competing more with the neighboring species for above and below ground resources, legume presence promoted the growth and N uptake of its neighbors via N sparing (Temperton et al. 2007; von Felten et al. 2009) or N transfer (Høgh-Jensen and Schjoerring 2000; Pirhofer-Walzl et al. 2012). Based on this, the true legume effect in our study might even exceed the observed facilitation. In addition, overall δ^{15}N values, leaf N concentration, shoot N uptake data of legume neighbors, and the legume species itself also support facilitation by the legume via N sparing or transfer, i.e. a legume effect. Thus, we conclude that the positive responses of neighboring non-legumes are due to legume facilitation rather than species-identity effects on competitive balance.

Patterns of legume facilitation effect on ANPP over time

We confirmed the second hypothesis that legume facilitation on ANPP of neighboring non-legumes increases over time. This significant effect was observed under control and heavy rain conditions (Fig. 6). The positive influence of legumes under drought also gradually increased with time, yet with a much smaller effect size (slope = 0.07). On sandy soils, productivity of temperate grasslands was found to be limited by N availability (Semmartin et al. 2007; Yu et al. 2009). Increasing importance of facilitation by legumes in the present study points towards a self-enforcing feedback of legume facilitation, which might be caused by increasingly efficient N-fixation in an N-poor sandy soil substrate. Roscher et al. (2011) reported increasing amount of N_2 fixation by legumes over four years after their establishment. Progressively positive legume effects on neighboring species over five years were observed by Marquard et al. (2009). Mulder et al. (2002) found less strong legume effects, but are also suggesting that legume presence contributes towards greater biomass and total nitrogen over the first three years in experimental grassland. However, most previous experiments were installed by sowing and thus could not investigate neighborhood effects between equally spaced individuals but only the overall performance of communities.

Effects of legume presence under drought

The third hypothesis that under annually recurrent drought legume neighbors would not have significantly higher ANPP, higher δ^{15}N, higher leaf N concentration, and higher shoot N uptake compared to non-legume neighbors, was only partly supported by the experiment. Facilitation of total ANPP of the three target species and of species specific ANPP of two of the three target species disappeared under drought conditions (Fig. 3). However, positive effects of legume presence found under control conditions persisted under drought conditions for ANPP of *A. elatius*, δ^{15}N of *P. lanceolata*, leaf N-concentrations, N-uptake, soil NH_4^+, and soil N% (Fig. 3-5, and Table 1). Possible reasons behind this reduced facilitation of growth can be either a reduction in N_2 fixation by legumes or an inhibition of N-uptake due to the water stress; but also to the interaction of both mechanisms.

Astonishingly, compared to the controls drought had no significant negative effect on total ANPP of our three target species. Drought was found to initiate plant physiological processes that can stabilize biomass production and this may regulate ecosystem productivity (Jentsch et al. 2011). Under drought, the positive legume effect on growth of the target species disappeared. This pattern could be explained by reduced N_2 fixation by legumes under drought. Serraj et al. (1999) showed the sensitivity of N_2 fixation to drought via impacts on nitrogenase activity, which depends on quantitative phloem flow into the nodules. Thus, any variation in phloem flow as a result of changes in turgor pressure in the leaf is likely to cause a change in nodule activity, which ultimately reduces of N_2 fixation under drought. In our experiment though, N_2 fixation by the legumes appeared not to be impaired in the drought plots, as indicated by the unaffected δ^{15}N, shoot N content, and leaf N concentration of the legume species itself (Fig. 7). Besides, we observed slightly higher soil N-concentrations in the drought plots. Here, we applied pulsed stress events and as a result could not detect if N_2 fixation had taken place during the peak of the drought treatment, as it could have occurred before and after the treatment. In addition, it is possible that higher soil N-concentrations observed in drought treatments could be driven by decaying roots after short-term partial die-back.

The lower leaf N concentration of both legume and non-legume neighbors grown under drought compared to those grown under control conditions emphasizes the limitation in N uptake by drought. At the species level, *H. lanatus* showed that leaf N concentration and shoot N uptake were significantly lower under drought conditions compared to control and legume presence could not compensate this reduced uptake (Table 1). A recent study found that N uptake efficiency is an important trait to support growth under drought (Weih et al. 2011). Abdelhamid et al. (2011a) found that dry surface soil causes reduction in shoot N uptake through reduction in N_2 fixation. Although the overall reduction of the legume effect under drought has already been documented, our study differs from previous approaches (Serraj et al. 1999; Sadale and Karadge 2010; Abdelhamid et al. 2011b; Nawaz et al. 2012) as well in the systematic design as according to the relevant mechanism by pointing at the N uptake (Abdelhamid et al. 2011a; Van den Berge et al. 2011) to be more sensitive to drought than N_2 fixation. Our reduced N-uptake results can be explained by limited diffusion of nutrients from the soil matrix to the absorbing root surface during low soil moisture (Hu et al. 2007). Consecutively, nutrient transport from the roots to the shoots decreases due to reduced transpiration rates (Duman 2012). Drought in our experiment was applied in forms of recurring pulsed stress events. Neighboring plants did not compensate the reduced uptake during the drought, although conditions before and after the drought manipulation allowed for N-uptake. In sum, the present study indicates that facilitation by a legume species is reduced under annually recurrent pulsed drought events as a consequence of reduced N-uptake rather than N_2 fixation.

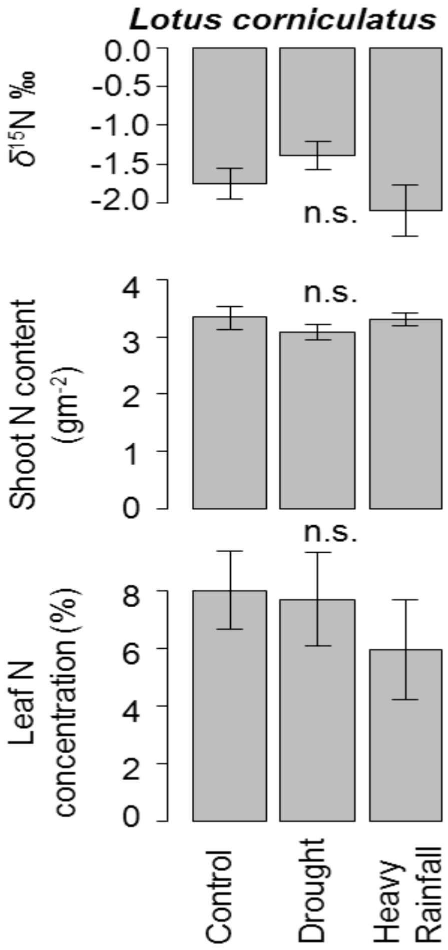


Fig. 7 Effect of climate manipulations on δ^{15}N, shoot N content, and leaf N concentration of the legume *L. corniculatus* (data from 2007). Data from 5 replications are shown.

Facilitation of growth by legume presence was differently affected by the drought manipulation for our three target species: *H lanatus* was facilitated only under control conditions, *P. lanceolata* was facilitated only under heavy rainfall, and *A. elatius* was facilitated irrespective of climate manipulations. The species differ in their rooting system with *P. lanceolata* being characterized by its persistent taproot system (similar to *L. corniculatus*, Klimešová and de Bello 2009) reaching deep into the soil and potentially providing an advantage compared to the shallow-rooted *H. lanatus* (Kutschera et al. 2009) in taking up N which is leached downward due to heavy rainfall and accompanied excess of water. Similarly, *A. elatius* is a species rooting deeper than *H. lanatus*

(Kutschera et al. 2009), while both are graminoids with dense, fibrous root system potentially being beneficial in comparison to *P. lanceolata* in the absence of excess water moving downward. The main message beyond this speculative explanation, however, is that facilitation under altered climatic conditions is highly species-specific. The three target species in our study showed complementary patterns, this finding can be linked to an overall buffering effect of biodiversity on ecosystem functioning in the face of perturbation (here extremes), as expected by the insurance hypothesis (Yachi and Loreau 1999).

Effects of legume presence under heavy rainfall

We finally hypothesized that under annually recurrent heavy rainfall legume neighbors would not exhibit significantly higher ANPP, higher δ^{15}N, higher leaf N concentration, and higher shoot N uptake compared to non-legume neighbors. This expectation was partially confirmed by our results (Table 1, Fig. 3, and Fig. 4). The total ANPP significantly increased with legume presence under heavy rainfall, while the corresponding values of δ^{15}N, leaf N concentration, and shoot N uptake did not show significant changes. A positive growth effect with increased precipitation can be either caused by increased N_2 fixation or by higher soil moisture and thus more nutrient uptake; but again it can also be a consequence of the interaction of both mechanisms.

Previous studies indicated that increased precipitation has positive effects on ANPP of grasslands (Sala et al. 1998, Yang et al. 2008), but such effects depend strongly on the regional climate and ecosystem. Temperate managed grasslands (*e.g.* hay meadows) cannot be directly compared to continental natural prairies. It is evident that adequate soil moisture supports N_2 fixation (Abdelhamid et al. 2011a, Guriqbal et al. 2011). Some legume species (i.e. *L. corniculatus*) can fix atmospheric N_2 even under waterlogged conditions when soil O_2 concentration becomes low (Witty et al. 1986; Pugh et al. 1995; James and Crawford 1998; Striker et al. 2005).

Although our heavy rain treatment had gone beyond field capacity towards water saturation (Fig.1), the δ^{15}N signature, shoot N content, and leaf N concentration of the legume species (*L. corniculatus*) did not differ significantly when comparing the heavy rainfall treatment with the control and the drought treatment. Besides, the interaction of legume presence and heavy rainfall had no significant effect on soil N status. Therefore, no evidence of higher N_2 fixation was found under heavy rain (Fig. 7). Increased N uptake or leaching under heavy rainfall could explain the absence of this response. Overall leaf N concentrations of legume neighbors were slightly higher under heavy rainfall compared to non-legume neighbors, indicating a beneficial effect of legume presence. The leaf N concentration of *H. lanatus* clearly reflected this beneficial effect. Unfortunately, our study cannot provide evidence for nitrogen leaching, but, it was found that a considerable amount of nitrogen losses can take place under low species diversity (Scherer-

Lorenzen et al. 2003), which we also have in the present study. Scherer-Lorenzen et al. (2003) observed increased leaching of nitrate in legume plots compared to non-legume plots. Here, we used a soil which is highly permeable. This soil type may have allowed leaching under heavy rain conditions. A previous study found that soil N leaching is associated with both heavy rainfall and soil type (Rimski-Korsakov et al. 2004). In the latter study, heavy rainfall even caused the leaching of previously accumulated soil nitrate. Therefore, it is possible that N_2 fixation may be enhanced in heavy rain plots, but this enrichment is not reflected in the plants because soil nitrogen is leached to the aquifer.

Conclusions

Legumes facilitated the productivity of neighboring plant species in experimental temperate grasslands. However, the positive legume effects on growth disappeared under extreme drought conditions and were generally species-specific in their response to climate manipulations. These findings additionally emphasize the importance of legume presence and species-specific traits or complementarity of species for maintaining a stable productivity in the face of altered climatic conditions.

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6.3 Manuscript 3

Title: Provenance-specific improvement of nitrogen status in grasses after drought combined with rewetting

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Key words: Climate change adaptation, Drying and rewetting, Extreme weather event, Ecotype, Grassland management, Plant physiology, Phenological stage, Precipitation manipulation experiments, Productivity.

Abstract

Extreme drought events can decrease N status of forage plants in grassland ecosystems by reducing soil N availability and plant N uptake. It has been suggested that drought combined with rewetting induce soil microbial activity, soil N mineralization and therefore increase soil N availability. Yet, it is unclear to which degree plants benefit from additional soil N availability after a drought combined with rewetting event. Here, we compared plant N status, i.e. N concentration, N content and C/N ratio of four temperate grass species. Particularly, we investigated populations grown from seeds from European provenances (four to six countries per species) after a drought treatment combined with rewetting (10 days harvest delay) versus continuously watered conditions for control. Our aim was to test how populations respond to drought, and to develop implications for its management adaptation in the course of climate change.

The experimental combination of drought and rewetting significantly increased plant N concentration (+96%), N content (+31%) and decreased C/N ratio (-46%) compared to control. Treatment effects significantly differed among target grass species. Population responses were provenance-specific. In 21 out of 24 possible regression analyses, provenance-specific responses after drought and rewetting were not related to climate at the location of origins. However, treatment effects on N content of *Arrhenatherum elatius* was related to precipitation seasonality, C/N ratio of *A. elatius* was related to summer heat moisture index and N concentration of *Festuca pratensis* was related to precipitation seasonality of origins. Populations from moist sites did not perform worse than presumably drought-adapted populations.

Harvest delay after drought is recommended as plant N status improved by rewetting. Improved plant N nutrition after drought and rewetting was related to higher N uptake, dilution effects and delayed plant maturation. Within species physiological responses to extreme drought event were provenance-specific. Yet, no general evidence of local adaptation was found. Therefore, it is likely that a mixture of populations can support the maintenance of plant N nutrition under drought.

Introduction

In recent years an increase in weather extremes was recorded [1,2]. Also in Europe, the probability of occurrence of extreme drought and heavy rainfall regimes is changing [3,4]. Besides an increasing likelihood for heat waves, also modified precipitation regimes are expected. This involves changes in annual precipitation, seasonality, intensity and variability of precipitation, and the frequency and duration of drought periods [5]. It is under debate, whether this also results in increased intra-annual variability because the scale of climatic fluctuations is difficult to project. Increased climatic variability may also lead to prolonged periods of extremely moist or dry conditions due to stable cyclone or anticyclone positions [6,7]. However, forecasts hint at generally more extreme weather conditions in the near future [8–10]. Even in areas that experience an increase in long-term average precipitation, periods of drought and higher variability in soil moisture is expected [5], which can affect plant responses.

Water is a fundament of life. However, the role of temporal variability in water availability, including periods of shortage and luxus, has rarely been addressed explicitly [11]. Evidence suggests that extreme drought can substantially alter ecosystem functions [12–15]. Besides the disastrous impact of severe drought, it is the modification of nutrient cycling and productivity caused by enhanced moisture variability that is of interest in managed ecosystems such as grasslands. Drought stress and associated low soil moisture reduce soil N availability by lowering microbial activity, microbial biomass N, N mineralization and nutrient diffusion in soils [16–18]. Drought also reduces total plant N uptake by decreasing transpiration and physical transport in plants [12,19–22].

Through these combined effects of moisture and nutrient availability, drought causes low plant productivity [3,13,23], but at the same time may increase plant nutritional quality (e.g. dilution effects; see [13,24]). Even if, strong resilience to drought of grassland community productivity was reported by Jentsch et al. [14], plant nutrient status and forage quality may be affected.

As many ecosystem processes are context-dependent (i.e. differ on substrates, in seasons, between species), rainfall events can also have positive effects on soil N availability [25,26], soil respiration [27], flowering phenology [28], plant facilitation [12], and productivity [29] in grassland ecosystems. Also for important single grass species such as *H. lanatus* improved plant N status after rainfall events and associated high soil moisture are reported [12].

Particularly during growing seasons the influence of recurrent drought and heavy rainfall and consequently increasing soil moisture variability must be understood. Combined drought and heavy rain event was found to increase root to shoot ratios in a North American prairie (Fay *et al.* 2003). Drought combined with rewetting supports temporary boost in soil microbial activity within few minutes or hours [25–27,30] and enhances soil N availability for several days that even exceeds the N availability of a regularly watered soil [26,31].

Grassland species exhibit opportunistic growth patterns, especially as far as Poaceae grasses are concerned, allowing leaf and root dieback during drought followed by quick growth of leaves and roots when rewetted [32,33]. Higher plant N concentration, N content and lower C/N ratio can therefore be expected after drought combined with rewetting due to stimulated plant N uptake resulting from higher soil moisture, higher soil N availability, and fluctuating water uptake. However, studies on the effects of drought combined with rewetting on plant N concentration, N content and C/N ratio are missing.

The performance of plant species during periods of environmental stress can depend on adaptations of local populations. Within a species’ range, individual populations experienced site-specific environmental history [34,35]. For instance, if a plant population was continuously confronted with extreme hygric conditions, selection processes will support the best adapted members. Additionally, short-term adaptive mechanisms such as ecological stress memory can be stimulated [36].

Kreyling et al. [37] found that geographic origin of grass species populations that is reflecting their past climatic experience can explain responses to late spring frost. Provenance-specific responses of grasses have also been reported from drought and warming experiments [23]. Within species, responses like seed germination, seedling recruitment, mortality, leaf number, flowering phenology, chlorophyll content and biomass production etc., are reported to differ due to adaptation to precipitation [35,38], temperature, [34,39] and aridity [40] at the location of origins.

However, there is an ongoing debate whether the selection of better-adapted populations can serve as an adaptation strategy in grassland ecosystems [37,41,42] as this approach has a potential risk that introduced populations can eradicate local populations and gene pools [43]. Yet, within-species differences in response to climatic extremes are rarely studied; in particular studies on within species plant N status variation after drought combined with rewetting are not available.

We compared the plant N status, i.e. N concentration, N content and C/N ratio, of four important grass species of European origin but with worldwide current distribution in managed grasslands [23]. Seeds from geographically distinct populations were grown in a common garden experiment and exposed to experimental drought combined with rewetting in order to identify intra-specific variability in responses. We hypothesized that (1) drought combined with rewetting will increase plant N concentration, N content and therefore decrease carbon to nitrogen ratio. We also hypothesized that (2) populations stemming from climates at the location of origins with frequent drought and rewetting events will exhibit adaptations to such conditions and outperform populations originated in more constant climates.

Materials and methods

Experimental site

The experiment was part of the EVENT III experiment [23] and was established in March 2009. It is located in Bayreuth, Germany, on the property of the Ecological-Botanical Gardens of the University of Bayreuth (49°55′19″ N, 11°34′55″ E). The regional climate is temperate and moderately continental, with mean annual temperature of 7.9 °C (1971–2000). The mean annual precipitation of 724 mm (1971– 2000) has a bimodal distribution with a major peak in June-July and a second peak in December-January [44].

Selection of species and populations

We selected four grass species i.e. *Arrhenatherum elatius*, *Alopecurus pratensis, Festuca pratensis and Holcus lanatus,* which are common in Central European managed grasslands and important contributors to biomass [45]. Besides local populations from Germany, we selected populations from Bulgaria, Hungary, Italy, Spain, and Sweden on the basis that the climates at the location of origins differ in terms of summer heat moisture index (i.e. an aridity index) and precipitation seasonality (Table 1). For *A. elatius* and *F. pratensis* populations were available from all regions. For *A. pratensis* and *H. lanatus* four regions were represented (Table 1).

Climatic parameters of geographic locations of origins

We looked for climatic parameters that characterize likelihood of drought combined with rewetting at the location of origins of each population. Unfortunately, only few climatic parameters are available to represent likelihood of drought combined with rewetting such as summer heat moisture index (SHM) and precipitation seasonality. SHM calculates growing season aridity and potential evapotranspiration conditions [46]. SHM = ((MWMT)/(MSP/1000)), where SHM, MWMT and MSP are the summer heat moisture index, mean warmest month temperature (°C), and mean annual summer (May to Sept.) precipitation (mm). Temperature and precipitation data were retrieved from the Worldclim database to a resolution of 5 arc-minutes [47]. The precipitation seasonality of origins is a bioclimatic parameter of the Worldclim database [47], which calculates the standard deviation of the monthly precipitation values expressed as a percentage of the mean of those values (i.e. the annual mean). These parameters, however, give only a rough estimate for the likelihood of drought combined with rewetting.

Table 1: Information of populations of *A. elatius*, *A. pratensis*, *F. pratensis*, and *H. lanatus* used in the experiment with location and others climatic parameters of origins. Populations were ordered by summer heat moisture (SHM) index. Higher values of SHM index and precipitation seasonality values indicate higher drought stress during growing seasons.

Population	Latitude	Longitude	Summer heat moisture index	Precipitation seasonality (Coefficient of variation)
A. elatius				
Germany	49°17'46"N	09°58'25"E	61.2	20
Hungary	47°12'00"N	17°52'00"E	75.5	26
Sweden	59°51'25"N	17°38'22"E	74.9	31
Bulgaria	42°00'00"N	24°50'00"E	85.7	21
Italy	44°55'12"N	09°44'46"E	104.2	26
Spain	40°50'07"N	00°47'55"E	131.4	41
A. pratensis				
Sweden	60°00'00"N	15°00'00"E	56.8	26
Germany	49°17'46"N	09°58'25"E	61.2	20
Hungary	47°12'00"N	17°52'00"E	75.5	26
Bulgaria	42°26'29"N	23°35'20"E	83.9	24
F. pratensis				
Spain	42°37'08"N	00°09'47"W	36.7	13
Italy	44°33'00"N	09°27'00"E	53.8	23
Sweden	60°00'00"N	15°00'00"E	56.8	26
Germany	49°17'46"N	09°58'25"E	61.2	20
Hungary	47°27'00"N	18°28'00"E	87.1	23
Bulgaria	42°19'05"N	23°45'07"E	94.4	23
H. lanatus				
Germany	49°17'46"N	09°58'25"E	61.25	20
Hungary	46°10'00"N	17°55'00"E	77.4	26
Italy	44°53'33"N	09°41'20"E	97.3	27
Bulgaria	42°31'19"N	24°48'52"E	99.3	25

Cultivation of the plants

The plants were cultivated from seeds at the branch office of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Poel, Germany, from February 2009 to April 2009. The seedlings were then transported to Bayreuth and individually planted into 4 L plastic pots filled with local topsoil. The soil substrate was sandy silt (pH = 7.27, total C = 1.89%, total N = 0.15%, plant-available NH_4^+ = 1.79 mg L^{-1}, plant-available NO_3^- = 22.50 mg L^{-1}). For the first two weeks after planting, the seedlings were watered generously with tap water to ensure growth. All individuals were then cut to a height of 7 cm in order to create equal starting conditions and exposed to ambient precipitation until the start of the experimental treatments on 27 May 2009. Biomass was harvested at the end of June 2009.

Experimental design

The potted individuals were exposed to two precipitation treatments (control vs. drought combined with rewetting) and to two temperature treatments (control vs. warming) in a split-plot design. The climate treatments were fully crossed, resulting in four climate manipulations (control, drought combined with rewetting, warming, warming + drought combined with rewetting), which were replicated three times, resulting in 12 experimental units in total. The population treatment was nested within each experimental unit.

Each population was further replicated with three individuals per experimental unit (nested replicates) resulting in nine replicates per population per treatment. The individual plants were assigned randomly to the experimental units for each species. Here, we focused on the overall responses to the precipitation and population treatment and used the individuals of the temperature treatments as additional replicates due to no considerable single and interactive effects of the temperature treatments (data not shown).

Each experimental unit was covered by a single rain-out shelter (GlasMetall RiemerGmbH, Rahden- Sielhorst, Germany) covered with transparent polyethylene sheet (0.2 mm, SPR5; Hermann Meyer KG, Rellingen, Germany). The lower edge of the rain-out shelters was at a height of 80 cm from the ground to support air circulation and to minimize greenhouse effects. The sheet permitted close to 90% penetration of photosynthetically active radiation.

The control precipitation regime simulated the local 30-year average daily precipitation (manipulation was done twice a week with collected rain water). The drought combined with rewetting treatment consisted of a period without precipitation and then rewetting the plants by the same amount of precipitation which was applied to the control plants during the drought manipulation. The definition of duration of drought treatment was based on sign of species-specific leaf senescence in >66% of the total individuals. A four-stage visual damage quantification key (0–3, where 0 stands for ‘completely undamaged’ and 3 stands for ‘totally dried out and brittle’) were used. By the time two-thirds of the individuals of one species had reached stages 2 or 3, or by the time one-third of the individuals had reached stage 3, the drought was stopped. The drought treatment lasted 16 days for *H. lanatus*, 18 days for *A. pratensis* and *F. pratensis* and 19 days for *A. elatius*.

The drought treatment resulted in a dropping of soil moisture below the permanent wilting point of the soil approximately one week after the start of the drought treatment (Fig. 1). In the rewetting phase, each individual plants of the drought treatment received 350 mL water on three consecutive days (1050 mL in total, this is the total amount of water which was applied to each individual of the control plants during the drought manipulation), which corresponds to 38 mm of precipitation. Rewetting of dry soil resulted in a steep increase of soil moisture (overall up to 28%, no detectable soil moisture variation observed between species). Then, the pots were watered according to the control precipitation treatment. Soil moisture after rewetting remained higher in the drought treated pots than in the control pots until we harvested the plants of both group together (10 days after the end of drought, respectively).

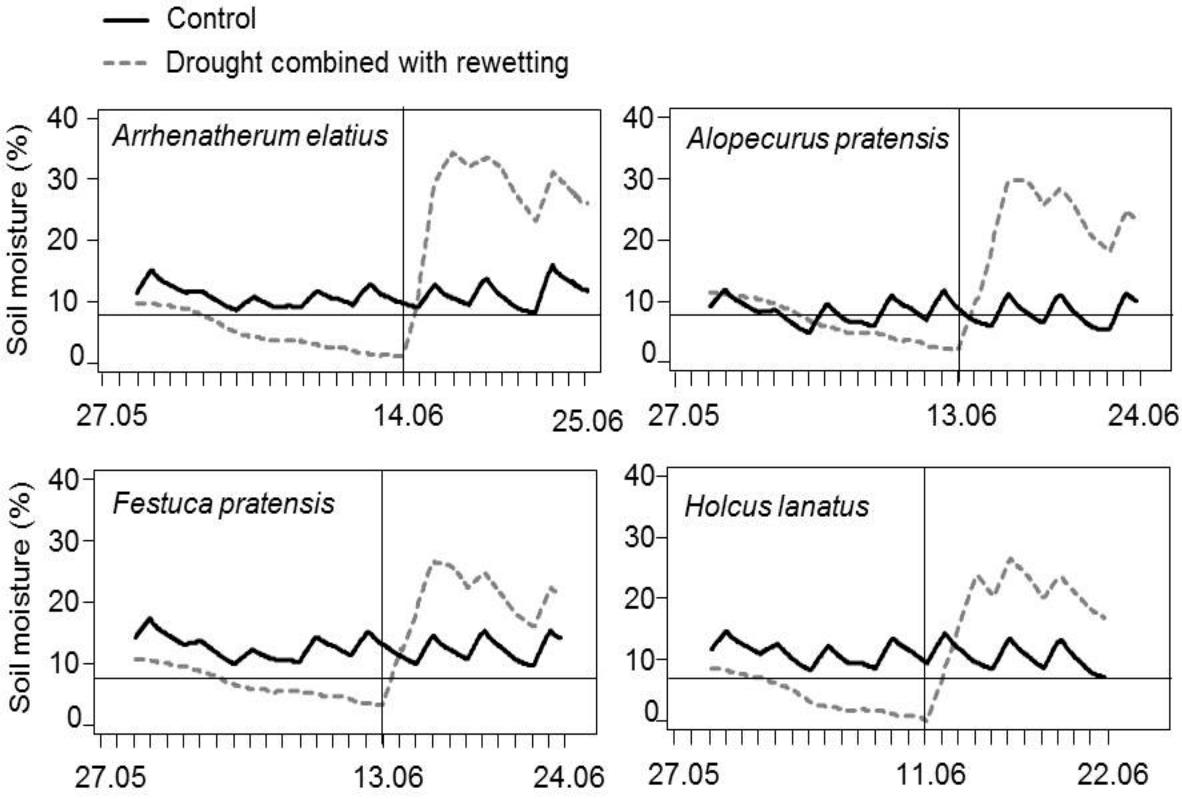


Fig. 1: Soil moisture at -2 to -7 cm soil depth during the experiment. Soil moisture was measured hourly by FD-sensors (Echo.EC-5 ⁄ k; Decagon Devices, Pullmann, WA, USA) at six randomly assigned pot for each species within each treatment. Mean values for each species are shown here. Precipitation manipulation was similar for all species and no detectable soil moisture variation observed between species. The horizontal line represents approximate permanent wilting point (pF = 4.2). The vertical line represents the end of drought and start of rewetting treatment.

Plant C and N status

The biomass harvest took place 10 days after the pulse drought manipulation ended, respectively, for each species, in order to account for the recovery of the plants. Each individual plant was cut at 3 cm above the soil and the biomass was dried for 48 h at 70 °C and weighed.

For plant C and N analysis, samples were randomly selected from above ground dry biomass including leaf and shoot as N concentration varies in different plant parts. Selected samples were fine-milled and mixed well for C and N analysis. Samples (3 mg approx.) were analyzed using an elemental analyzer (EA 3000; Euro Vector, Italy). Relative plant C and N concentrations (%) were provided by this analysis. Total plant N content (g/plant) was calculated by multiplying the total above ground dry biomass by the respective plant N concentration.

Statistics

Data were analyzed in two phases. In the first phase, linear mixed-effect models (LMEMs) combined with analysis of variance (ANOVA) were applied to test for the main and interactive effects of the two factors: species and precipitation treatments (individuals of temperature treatments used as additional replicates in the present study, see experimental design for details). The split-plot design and the nested replicates were accounted by the use of the experimental unit identity as a random factor. Population identity was additionally included as random factor in this analysis. In the second phase, LMEMs combined with ANOVA were applied for each species separately to test for the main and interactive effects of population and precipitation treatments. Here, experimental unit identity was used as random factor. Homogeneous groups of factor combinations were identified by Tukey's HSD post hoc comparisons. Level of significance was set to $p < 0.05$. Data were transformed by square-root to improve the homogeneity of variances and the normality of residuals prior to analysis, if necessary. All characteristics were tested by examining the residuals versus fitted plots and the normal qq-plots of the linear models.

Regression analysis between relative responses of populations to the drought combined with rewetting and climate at the location of origins were conducted with linear least-squares regression (function 'lm'). Relative responses of populations were calculated from the mean difference between drought combined with rewetting and control divided by control. LMEMs were conducted with the function 'lme' and Tukey HSD post hoc comparisons by the function 'glht'. The three packages multcomp (Simultaneous Inference in General Parametric Models, version:1.3-2), lme4 (Linear mixed-effects models using Eigen and S4, version: 1.0-6) and sciplot (Scientific Graphing Functions for Factorial Designs, version: 1.1-0) were used for analysis and graph preparation in R version 3.0.2 [48].

Results

Drought combined with rewetting significantly increased overall plant N concentration (+96%) and N content (+31%) and decreased carbon to nitrogen ratio (-46%) of four grass species compared to control (Fig. 2). Treatment effects on plant N status significantly differed among species. The increase in plant N concentration after drought combined with rewetting treatment did not differ significantly between *A. pratensis* and *F. pratensis* (Ap: Fp, *p* = 0.9659), however, they performed better compared to both *A. elatius* and *H. lanatus* (Ap : Ae, *p* = 0.0190; Ap : Hl, *p* = 0.0001; Fp : Ae, *p* = 0.0001; Fp : Hl, *p* = 0.0001). Among those, *A. elatius* showed significantly higher plant N concentration than *H. lanatus* (Ae : Hl, *p* = 0.0128). Drought combined with rewetting compared to control also resulted in significantly higher plant N content of three grass species, *A. pratensis, F. pratensis, and H. lanatus,* but not of *A. elatius* (Fig. 2). No interaction between species and drought combined with rewetting was observed for plant C/N ratio (Fig. 2).

Drought combined with rewetting led to provenance-specific (four to six European population per species) plant N status. Plant N concentration differed significantly among populations of *A. elatius, F. pratensis* and *H. lanatus*. This was not found in *A. pratensis* (Table 2, Fig. 3). However, total plant N content and C/N ratio differed significantly among the populations of all four grass species (Table 2, Fig. 3).

The relative treatment effects of populations were not found to be significantly related to climate at the location of origins in 21 out of 24 possible regression analyses (4 species × 3 response parameters × 2 climatic parameters of origins). However, the relative treatment effects on N content of *A. elatius* was marginally related with precipitation seasonality (r^2 = 0.57, *p* = 0.0495), C/N ratio of *A. elatius* was related with SHM index (r^2 = 0.71, *p* = 0.0216), and N concentration of *F. pratensis* was marginally related with precipitation seasonality (r^2 = 0.57, *p* = 0.0497) (Supporting information S1). Surprisingly, a probably drought-adapted population from Spain performed lower than local German population for N content of *A. elatius* (post-hoc test: Spain vs. German, *p* = 0.0001). However, no significant differences were detected between a probably drought-adapted population and the local population that has evolved under temperate climate for C/N ratio of *A. elatius* and N concentration of *F. pratensis* (post-hoc test: C/N ratio of *A. elatius*, Germany vs. Spain, *p* = 0.9938; N concentration of *F. pratensis*, German vs. Sweden, p = 0.9999). Relative treatment effects on plant N status of *A. pratensis and H. lanatus* were not related with SHM and precipitation seasonality (Supporting information S1).

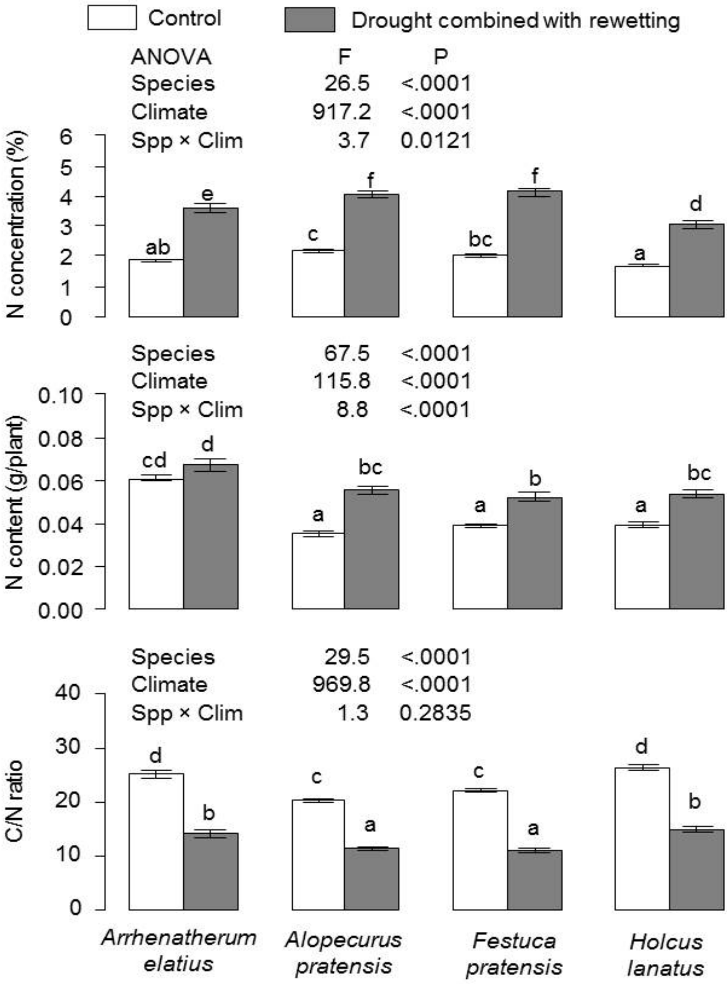


Fig. 2: Effects of climate treatments on plant N concentration, N content and C/N ratio of four grass species. Mean values and standard errors are shown in the figure. Significant treatment effects are indicated by different letters ($p < 0.05$).

Discussion

We confirmed the hypothesis that drought combined with rewetting will increase plant N concentration, N content, and therefore decreases carbon to nitrogen ratio. The overall increase in plant N concentration, N content and decrease in C/N ratio might be due to higher N uptake resulting from increased soil N availability after drought combined with rewetting.

Previous studies indicate that plant N uptake and plant N status linearly increases with soil N availability [49,50]. Grassland species can increase their N uptake rate by 70% and overall plant N concentration by 40% if soil N availability is increased by around 120% [50]. It has been reported that rewetting of dry soils increases N mineralization over 300% [51] at least for short periods of time. This can enhance soil N availability dramatically for several days [26,31]. Our drought treated grass species benefited in N uptake too, as indicated by 96% higher plant N concentration, 31% higher plant N content and 46% lower plant C/N ratio.

Table 2: Responses of European populations of four grass species after drought combined with rewetting. ANOVA results of the applied mixed models are shown here. Analyses were run for each species and response parameter separately. Displayed are the degrees of freedom (d.f.), F- and P-statistics.

	Arrhenatherum elatius				*Alopecurus pratensis*				*Festuca pratensis*				*Holcus lanatus*			
	Num d.f.	Den d.f.	F	P	Num d.f.	Den d.f.	F	P	Num d.f.	Den d.f.	F	P	Num d.f.	Den d.f.	F	P
N concentration (%)																
Population	5	149	5.3	0.0002	3	126	1.7	0.1812	5	146	4.4	0.0009	3	108	2.7	0.0519
Treatment	1	149	190.8	<.0001	1	126	270.4	<.0001	1	146	529.6	<.0001	1	108	174.4	<.0001
Population × Treatment	5	149	5.2	0.0002	3	126	0.6	0.6032	5	146	11.1	<.0001	3	108	0.4	0.7444
N content (g/plant)																
Population	5	149	6.2	<.0001	3	126	3.2	0.0262	5	146	4.5	0.0008	3	108	3.7	0.0143
Treatment	1	149	1.6	0.2132	1	126	97.6	<.0001	1	146	46.7	<.0001	1	108	51.8	<.0001
Population × Treatment	5	149	2.2	0.0563	3	126	1.3	0.2744	5	146	4.0	0.0021	3	108	1.5	0.2135
C/N ratio																
Population	5	149	4.5	0.0007	3	126	3.1	0.0277	5	146	4.8	0.0004	3	108	6.1	0.0007
Treatment	1	149	143.1	<.0001	1	126	331.4	<.0001	1	146	356.8	<.0001	1	108	215.1	<.0001
Population × Treatment	5	149	3.6	0.0043	3	126	1.8	0.1600	5	146	6.8	<.0001	3	108	0.7	0.5719

Plant N concentration response to drought stress can vary due to dilution effects (increased N concentration with decreasing biomass). The tendency of plant N concentration to increases with decreasing plant biomass due to the higher ratio of metabolic versus structural tissue that contain little N [52]. Dilution effects were evident in the present study as indicated by lower biomass production (-29%) after drought combined with rewetting compared to control (Fig. 4).

Albert et al. [32] found that the change in the C/N ratio of a grass species (*Deschampsia flexuosa*) under drought is governed by the differences in plant N concentrations. This is supported in the present study by higher plant N concentration and lower C/N ratio. However, higher total plant N content after drought combined with rewetting in the present study suggested that there might be additional factors such as higher soil N availability and plant N uptake, which enhanced N status of the grasses. This assumption is in accordance with Sardans et al. [21] who found that increases in plant N concentrations under drought is not only resulting from dilution effects but also from modified soil N availability, photosynthetic capacity, and plant internal C and N remobilization.

Plant N status is known to change with age. Young leaves are richer in N than older ones [53,54]. It has been reported that mild to moderate drought stress delays plant maturation [53,54]. In total, 16 to 19 days drought stress in the present study have slowed down the plant maturity as indicated by phenological responses. In total, 12% less individuals reached the phenological stage “start of inflorescence elongation” under drought combined with rewetting compared to control (Fig. 5). Furthermore, we visually observed that drought treated plants developed more new leaves during the rewetting period compared to control plants. These two processes, i.e. delayed plant maturation and stimulated development of new leaves after drought combined with rewetting, can be important response mechanisms in the face of increased climatic variability.

Albert et al. [32] suggest that a drought affected grass species (*Deschampsia flexuosa*) can reverse eco-physiological responses through quick re-growth of active tissue when rewetted. We found in addition that drought combined with rewetting increased plant N nutrition of grasses compared to continuously watered control conditions. Furthermore, the response in N status differed among grass species.

We showed that intra-specific variability (between phenotypes or populations of the same grass species) can contribute strongly to experimental responses in N-status and forage quality when climatic treatments are performed. Significantly higher N concentration in *A. elatius* compared to *H. lanatus* after drought combined with rewetting were influenced by populations of *A. elatius* from Sweden and Hungary, both performing better compared to the all four populations of *H. lanatus*. However, higher N concentrations of *A. pratensis* and *F. pratensis* compared to both *A. elatius* and *H. lanatus* after drought combined with rewetting were due to dilution effects as indicated by lower biomass productions. Drought combined with rewetting had no significant effect on plant N content in *A. elatius* compared to the control. Differences in plant N status between species can also be due to specific-specific N uptake ability [55].

The second hypothesis that populations stemming from climates at the location of origins with frequent drought and rewetting events will exhibit adaptations to such conditions and outperform populations originated in more constant climates is not supported by the present study.

Although we found differences between European populations of the selected grass species in plant N concentration (except *A. pratensis*), plant N content and C/N ratio, this was not a general pattern across all species and there was no geographic pattern that could be correlated with climate variability at the location of origin. The first aspect confirms the study of Beierkuhnlein et al. [23] where *A. pratensis* also did not show considerable within-species variability in response to extreme climatic conditions in contrast to other grasses. The second point can be due to insufficient geographical coverage and sample size (number of populations).

The two significant correlations between C/N ratio of *A. elatius* with SHM index and N concentration of *F. pratensis* with precipitation seasonality were influenced by dilution effects as indicated by no significant correlation of N content of *A. elatius* with SHM index and N content of *F. pratensis* with precipitation seasonality. The correlation between N content of *A. elatius* with precipitation seasonality showed that the presumably drought-adapted population from Spain performed even worse than the local German population. Beierkuhnlein et al. [23] found similarly that biomass production of the local German population did not perform worse than the population stemming from a drought-exposed location. This suggests that experimentally simulated climatic extremes did not exceed the phenotypic plasticity of local grass populations. In such cases, assisted migration of southern provenances [41] are not needed to maintain ecosystem functioning.

In another long-term drought manipulations experiment, it has been found that local grass populations can be adapted to an extreme drought events with 1000-years likelihood of recurrence (i.e. 42 days drought, see Jentsch et al. [14]). However, we compared relative treatment effects on plant N status of populations with two selected climatic parameters (SHM index and precipitation variability) and found no significant correlation in 21 cases out of 24 regression analysis. This might be due to the unavailability of right climatic parameters which represent the likelihood of recurrent drought and rewetting at the location of origin. Moreover, the lower sample size (four to six European sites only) and therefore selection of populations within a narrower climatic gradient could also be the reason for no general pattern observed here.

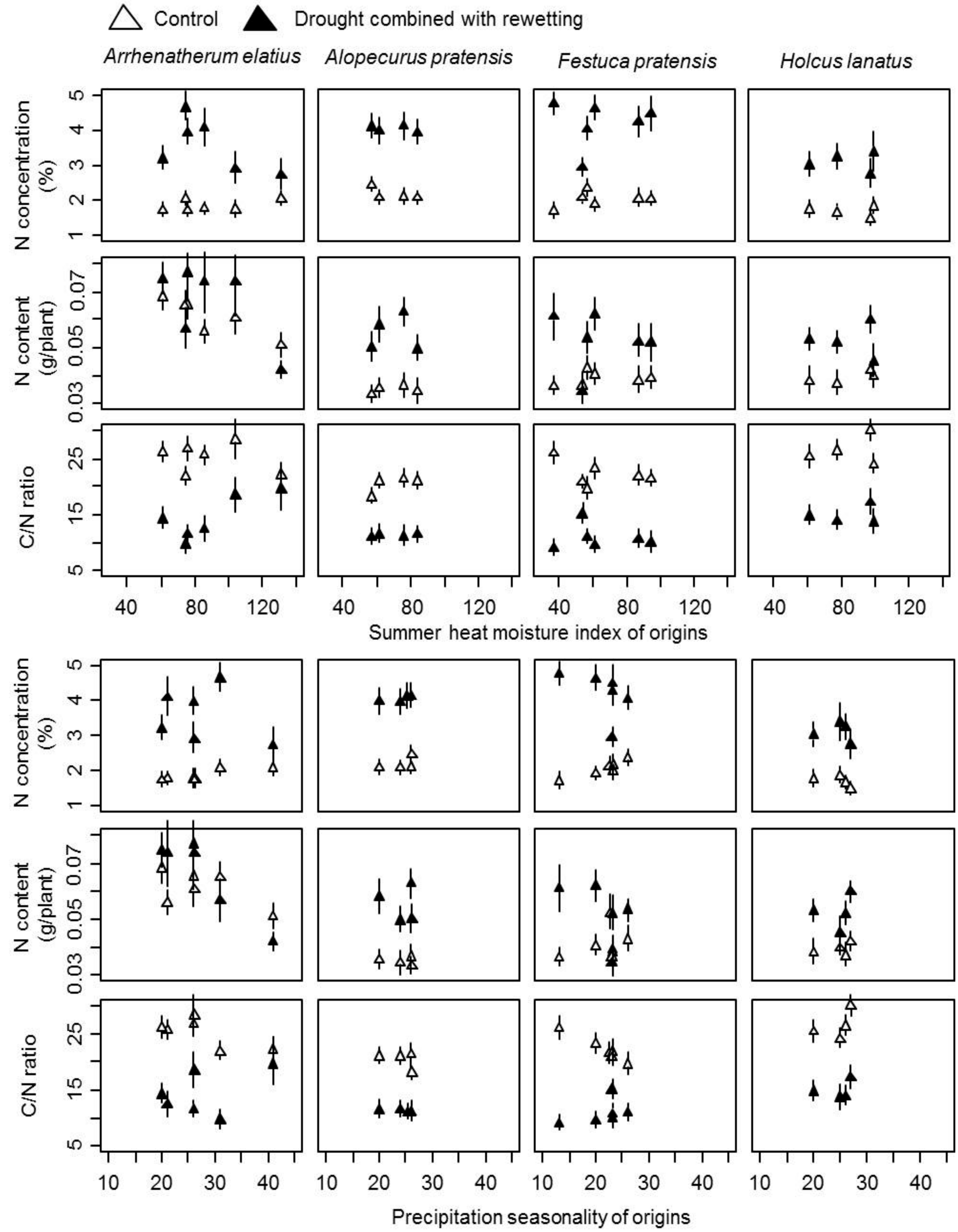


Fig. 3: Responses of four grass species and their selected European populations after drought combined with rewetting treatment. Mean values and standard errors are shown in the figure.

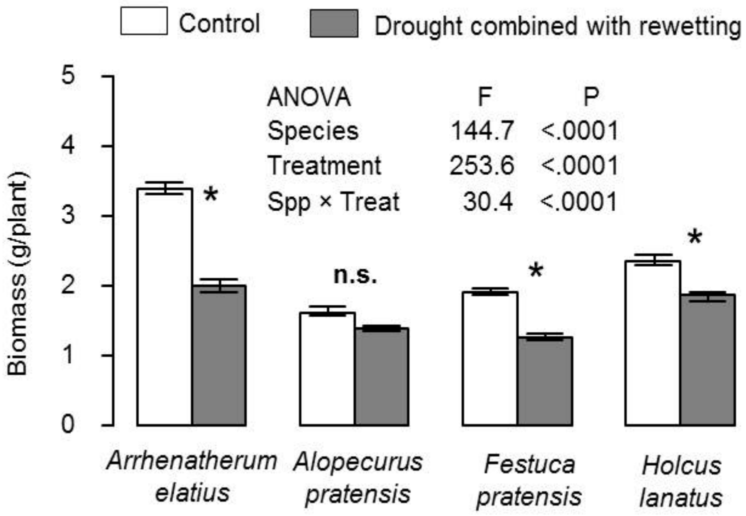
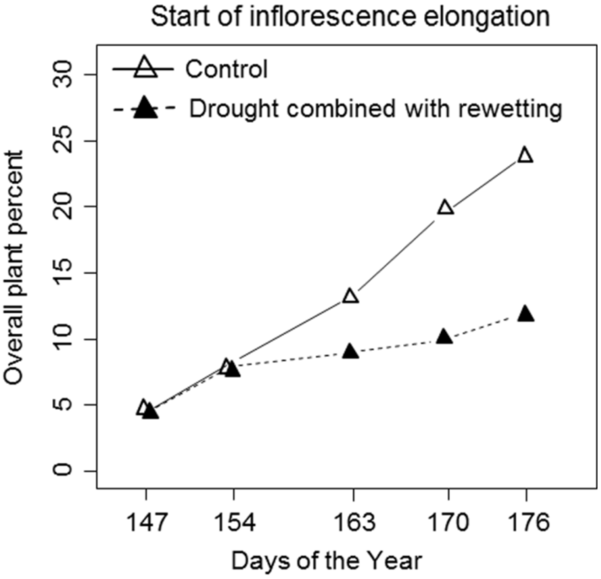


Fig. 4: Effects of climate treatments on above ground biomass production of four major European grass species. Mean values and standard errors are shown in the figure. Significant treatment effects are indicated by asterisk ($p < 0.05$).

Fig. 5: Effects of climate treatments on a phenological stage "Start of inflorescence elongation" of grass species. Mean responses of four grass species are shown here. Percentage of individuals reaches that stage (before the harvest) are shown in the figure. The documentation of the phenological stage began at the start of the drought treatment and continues until the biomass was harvested. Data were collected ones a week (in total 5 weeks).



Implications and Outlook

The present study highlights the importance of timing of forage harvest in grasslands in the face of climate change and increased climatic variability. It is most likely that many European grassland ecosystems will face more frequent changes between drought and heavy rain events in the growing season [4,5,8–10,12,13].

We manipulated drought stress and harvested the biomass after 10 days of a following rewetting event, where biomass was reduced by 29% compared to control (both harvested at the same time), but plant N status improved significantly (N concentration, +96%; N content, +31%, and C/N ratio, -46%).

The present study could not provide the response variations in biomass production and plant N status by drought (16 to 19 days) and rewetting separately (10 days) as we did not harvest the plants at the end of drought rather we captured the total combined effects of drought and rewetting. However, in a recent study by Grant et al. [13], it is reported that 10 days of rewetting after drought do increase biomass production. Therefore, if a drought event is peaking during the growing season, a potential management strategy would be harvesting grassland biomass at least 10 days after a following rain event. Yet, the present study showed that harvest delay would not compensate the total negative effects of drought on biomass production, but would increase plant N nutrition.

In addition, population responses were provenance-specific. Therefore, it is expected that a mixture of populations can support the maintenance of plant N status in the face of extreme drought events. However, before doing so, a broader debate should be started elucidating negative and positive effects of populations' mixture. If local grass species populations are already (or still) genetically diverse, this is the best way of insuring a diversity of responses and compensation within species. Such genetically diverse populations can be expected in old-grown grasslands. Ploughing and seeding grasslands with defined populations is in contrast to this and can yield increased risks in the face of climate change even if these populations are performing optimum under average conditions.

Conclusions

We conclude that drought combined with rewetting improved plant N status (i.e. higher N concentration, N content and lower C/N ratio) of four grass species due to higher N uptake compared to control conditions. Within each of the four grass species, populations stemming from different parts of Europe significantly differed in their response to drought combined with rewetting which indicates that the populations grown from certain accessions may represent ecotypes. However, presumably better drought-adapted populations did not outperform other populations or the local population after drought combined with rewetting. Therefore, it remains still unclear whether the selection of better-adapted populations could serve as an adaptation strategy in grassland ecosystems.

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Author Contributions

Conceived and designed the experiments: CB, AJ, JK. Analyzed the data: MASAK. Designed Figures and tables in the manuscript: MASAK. Wrote the paper: MASAK. Interpretation of results: MASAK, JK, CB, AJ.

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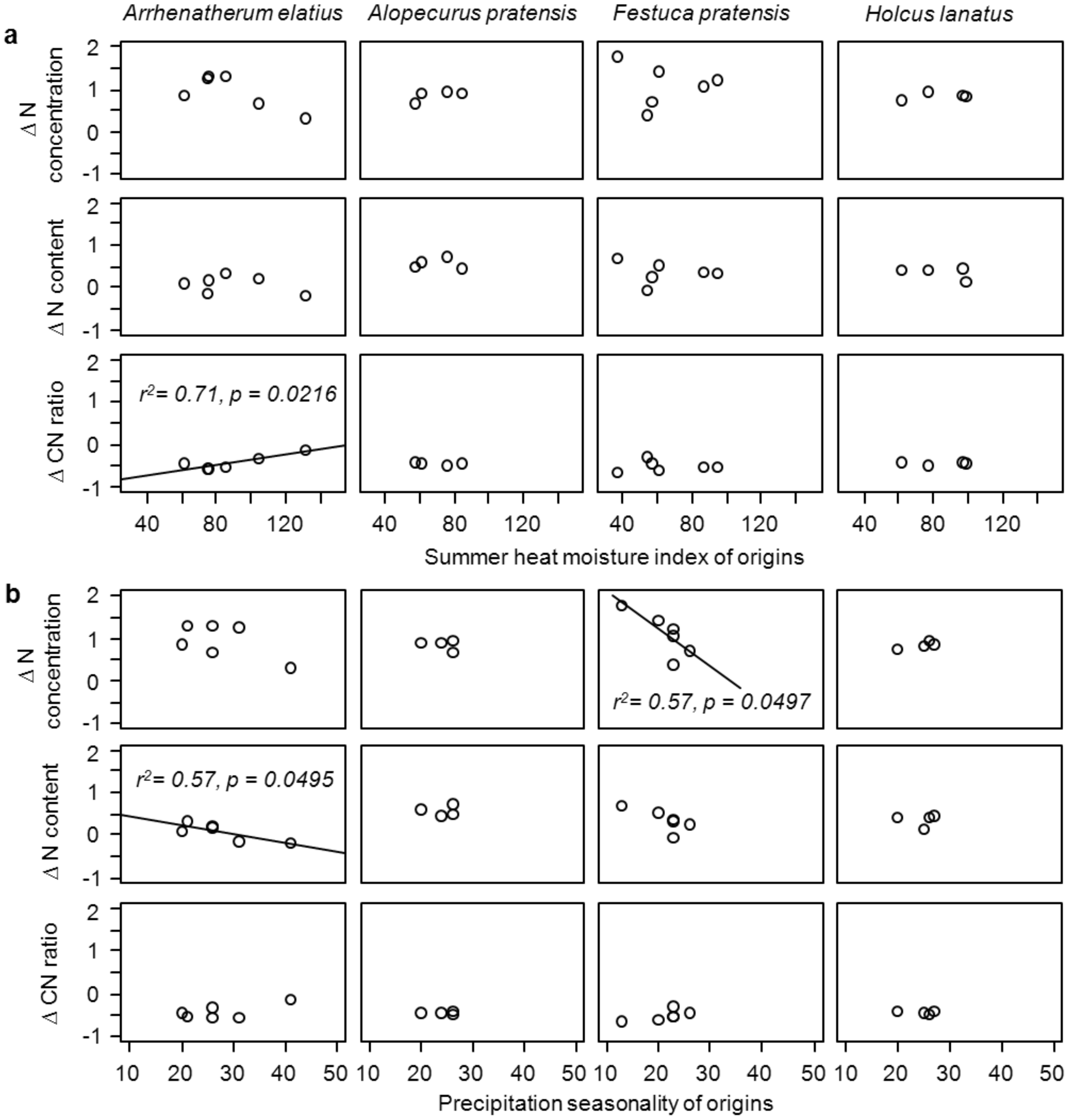
Supporting information S1: Regression between relative responses {(treatment – control)/control} of European populations of four grass species and climate at the location of origins (a) summer heat moisture index and (b) precipitation seasonality (Coefficient of variation). Regression lines are shown for significant correlations ($p < 0.05$).



6.4 Manuscript 4

Title: Factors influencing seedling emergence success of three global invaders under representative climates of seven biomes

Journal: Perspectives in Plant Ecology, Evolution and Systematics (In preparation to submit in Mid November 2015)

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Keywords: Climate change, Biological invasion, Cross-continental range expansion, Ecosystem vulnerability, Invasive plant, Provenance.

Abstract

The germination and seedling emergence successes in new environments are crucial first steps in life history of global plant invaders, which play key role into processes of range expansion.

We examined the germination and seedling emergence success of three global plant invaders - *Lupinus polyphyllus, Senecio inaequidens, and Verbascum thapsus* - in greenhouse and climate chamber with climate regimes corresponding to seven biomes (here after “experimental-climates”). Seed materials were collected from one non-native population for *L. polyphyllus* and *Senecio inaequidens*, and from 12 populations for *V. thapsus* (six native and six non-native).

Experimental-climates have significant effects on responses of three species. No germination and seedling emergences of any species were observed in one dry (humidity $\leq$ 50%) and in one cool (temperature $\leq$ 5°C) experimental-climate. However, all species germinated and emerged in two moderate cool (12-19°C) and in three warm experimental-climates (24-27°C). *V. thapsus* showed higher plasticity than *S. inaequidens* and *L. polyphyllus*. Non-native populations of *V. thapsus* steaming from warmer seed region showed higher performance in three warm experimental-climates and lower performance in two moderately cool experimental-climates than native populations. Responses of *V. thapsus* populations were related to temperature and precipitation of seed region in moderately stress experimental-climates (cool or dry).

The warm and semi-arid to humid experimental-climates are suitable for crucial first steps of invasion success by *L. polyphyllus*, *S. inaequidens*, and *V. thapsus*. Phenotypic plasticity modifies responses of three species under different experimental-climates. Climate of seed region influences responses of native and non-native populations of *V. thapsus*.

Introduction

Germination and seedling emergence are key traits in the life-history of plants, which contribute to the invasion success in new habitats (Beckmann et al., 2011; Brändle et al., 2003; Donohue et al., 2010; Hierro et al., 2009; Hirsch et al., 2012; Luo and Cardina, 2012). Plants introduced in new habitats often face contrasting climates in comparison to their origin. Therefore, the ability to germinate under a wide range of climatic conditions and adaptation to climatic stress during the seedling emergence stage is crucial for the establishment success of invaders (Cervera and Parra-Tabla, 2009; Hou et al., 2014). Even after the establishment of an invader in a new environment, germination and seedling emergence success are important because they facilitate further range expansion processes (Theoharides and Dukes, 2007). Moreover, the environmental conditions under which germination takes place determines the environmental conditions experienced in later life stage (Donohue et al., 2005).

Despite the several previous studies on why and how global invaders respond to new environments (Alba and Hufbauer, 2012; Ebeling et al., 2008; Kumschick et al., 2013), only few of them addressed the question of where and why they can or cannot germinate (Hou et al., 2014), grow and establish themselves (Alexander et al., 2012). Phenotypic plasticity would permit predictions on the future range expansion of global invaders (Bossdorf et al., 2005; Parker et al., 2003). The range expansion becomes predictable, if high plasticity lets the species thrive in many different climatic conditions or if the species is neither plastic nor fast evolving, but just fill its climatic niches in new places. In these conditions, contingency plans for threatened areas can be set up by experimental studies. If, on the other hand, range expansion is primarily guided by rapid adaptation or genetic variations (Alexander et al., 2012; Lachmuth et al., 2010), then the prediction is much more difficult to make. Such species may germinate and adapt to completely novel circumstances (Hierro et al., 2009) and establish in unexpected places (Kumschick et al., 2013; Maron et al., 2004; Prentis et al., 2008). It is important to test which biomes would be at risk in the near future, as the species could quickly adapt to the climates of new ranges outside of their actual distribution.

Predicting the future range expansion and understanding the vulnerability of various biomes to plant invasion are two important ecological issues facing the world today. Exploring the climatic thresholds for various life-history traits of global invaders species can help predicting their future range expansion (Alexander et al., 2012; Beckmann et al., 2011; Luo and Cardina, 2012). Width of germination niche alone can strongly influence ecological or geographic ranges of plant species (Brändle et al., 2003; Cervera and Parra-Tabla, 2009; Grime et al., 1981; Hierro et al., 2009) as germination is frequently subjected to natural selection before other life-history traits are expressed (Donohue et al., 2005). Distributional patterns of plant species can also be affected by the environmental conditions essential for seedling emergence and establishment (Donohue et al., 2010). It would therefore be worthwhile to examine climatic thresholds, explore the climatic niche for germination and seedling emergence of global invaders, and predict their possible future geographic range based on it. Up to now, the climatic thresholds of germination and seedling emergence success of global invaders have rarely been tested across species under various contrasting environments (Hou et al., 2014).

Furthermore, studying germination and seedling emergence of native and non-native populations of a global invader can improve our understanding of the processes of range expansion (Donohue et al., 2010). Studies done under a few climatic conditions generally conclude that non-native populations have higher germination responses than native ones (Beckmann et al., 2011; Hierro et al., 2009; Hirsch et al., 2012), which might contribute to the establishment success of globally distributed invaders. The temperature and precipitation of seed region can influence establishment success of global invaders because germination responses of native and non-native populations differs along their corresponding climatic gradient (Eckhart et al., 2011; Hirsch et al., 2012). For instance, Eckhart *et al*. (2011) found that germination patterns of 20 populations of *Clarkia xantiana* were related to the temperature and precipitation of seed region. Hirsch et al. (2012) showed that precipitation of seed region has negative effects on germination response of native and non-native populations of *Ulmus pumila*. Therefore, for more insights about the ongoing range expansion of a widely distributed invader, it is essential, as already suggested by previous studies (Beckmann et al., 2011; Donohue et al., 2010; Hierro et al., 2009), to examine germination and seedling emergence success of its native and non-native populations under different climatic conditions. This kind of experiment will allow us to differentiate whether the germination and seedling emergence success of native and non-native populations are influenced by local adaptation or by phenotypic plasticity.

Here, we first tested germination and seedling emergence rates of three global invaders, namely *Verbascum thapsus, Lupinus polyphyllus, and Senecio inaequidens* under seven experimental-climate conditions representing gradients in temperature and moisture availability. Our null hypothesis is that the seedling emergence of three global invaders will not be hindered by any of the seven experimental-climate conditions, with all three species having consistent performance. Secondly, the performance of six native and six non-native *V. thapsus* populations (two native and two non-native continents collected at three different seed regions each) were also investigated under seven experimental-climate conditions. We hypothesized that performances of populations from native and non-native ranges will be determined by local adaptation. In this context, we expected that the climates of seed regions (temperature and precipitation of seed source) are related to responses of native and non-native populations of *V. thapsus* under experimental-climates.

Methods

Study species: invasive across continents

Lupinus polyphyllus (Fabaceae), the Garden Lupin, is native to Western parts of North America (California, Alaska, west Oregon, and west Washington) and Canada (British Columbia). Since the 1900s it has been introduced in many European countries including Austria, Denmark, England, Estonia, Finland, Germany, Italy, Latvia, Lithuania, Norway, Poland, Sweden and the central part of European Russia as an ornamental plant, for soil improvement and stabilization, and as fodder (Fremstad, 2010). The species typically strive in moist habitats but the main invaded habitat types are nutrient-limited acidic grasslands in upland region.

Senecio inaequidens (Asteraceae), the South African Ragwort, is native to South Africa but has been introduced in Europe, especially in Belgium, France, Germany, Italy, Netherlands, Scotland, and Spain during the late 20th century. The species reached the Mediterranean coast, and expansion continued towards the Eastern Pyrenean region. *S. inaequidens* also invaded many habitats of South America (Ernst, 1998). It has colonized a wide range of disturbed habitats including fallow ground, river banks, rocky habitats, coastal dunes, heavily grazed and recently burned grasslands, and it occurs along traffic routes such as roads and railways (Lachmuth et al., 2010).

Verbascum thapsus (Scrophulariaceae), the common mullein, is native to Eurasia. Its geographical distribution ranges from Western Europe to China, crossing to the east in Russia. It is also present in Turkey where the genus of *Verbascum* is currently composed of more than 250 species (Alba and Hufbauer, 2012; Sharifnia, 2007). *V. thapsus* now exists in Canada, in all states of the USA, Australia, New Zealand and La Reunion (Ansari and Daehler, 2010). It is considered as a noxious weed in Colorado, South Dakota, and Hawaii (Alba and Hufbauer, 2012) and is one of the most common species in mountain regions around the world (Seipel et al., 2015, 2012).

Collection and storage of seed material

Seeds of *L. polyphyllus* and *S. inaequidens* were collected from non-native European provenances in Germany. Seeds of *V. thapsus* were collected from two native continents in Europe (Switzerland) and Asia (India), and from two non-native continents in North America (Montana/Oregon), and Australia (New South Wales). From each continent, populations of three different seed region (in total 12 populations) that differs by local climatic conditions such as mean annual temperature and annual precipitation were collected (Table 1). We focused on differences in mean annual temperature and in annual precipitation in the seed region, as it is evident that temperature and precipitation are important drivers of germination and establishment success of many invasive species (Alexander et al., 2012; Beckmann et al., 2011; Elliott et al., 2011;

Kumschick et al., 2013; Monty and Mahy, 2009; Wang et al., 2009). Overall, mean annual temperature of seed regions was warmer for non-native than for native populations (Table 1). In contrast, the overall precipitation of seed regions was significantly higher for native than for non-native populations of *V. thapsus*. The mean annual temperature and annual precipitation ranges from 2.4 to 15.3 ºC and 388 to 1353 mm respectively among the seed regions of *V. thapsus* populations (Source: Worldclim, Hijmans, Cameron, Parra, Jones, & Jarvis, 2005). After seed collection in the year 2009, all seeds used in the present experiment were stored in a refrigerator at temperatures below 0 ºC.

Germination trials in various experimental-climates

Seeds were placed in petri dishes (9.0 cm diameter, 20 seeds/dish, n = 5 for all factorial combinations) containing Rotilabo round filter paper (601A, cellulose, Ø membrane 90 mm). Petri dishes were initially sprayed once with fungicide (Previcur N Fungicide, diluted to 0.15% per liter) to prevent the seeds from fungal attack, and then placed in seven experimental-climates (see below for details). From the following days of the experiment, the dishes were watered daily (if needed) between 9 and 11 am to keep the filter paper moist. Water level and photoperiod have significant effects on germination and seedling emergence rates (Wainwright and Cleland, 2013). Therefore, we used the same water level (5 ml/petri dish/day) and approximately the same photoperiod (13-15 h day/9-11 h night) in all experimental setups to disentangle the effects of experimental-climates (mainly temperature and humidity) on germination and seedling emergence.

Experimental-climates corresponding to seven biomes

We investigated seven experimental-climates, five in greenhouse conditions and two in climate chambers. The facilities of the botanical garden at the University of Bayreuth were used as proxy for experimental-climates representing the following biomes: Semi-Arid Mediterranean, Semi-Arid Sub-Tropical, Humid Sub-Tropical, Semi-Arid Tropical-Alpine, and Humid Tropical. We therefore named these experimental-climates accordingly. Plants inside the greenhouses were mostly grown in climatic conditions similar to their natural biomes (Figure S1), for instance, the Semi-Arid Mediterranean greenhouse was the home for trees and shrubs of the Mediterranean zone: Citrus-trees from the Mediterranean, Myrtle plants from Australia (Callistemon- or Eucalyptus species) or palms from Asia and North America. In the Semi-Arid Sub-Tropical greenhouse water storing (succulent) plants such as cacti and many representatives of the Euphorbiaceae and Apocynaceae families are extensively managed over the whole year. The Humid Sub-Tropical greenhouse environment is little colder and mistier, accommodating several endemic plant species of the laurel forests of the Canary Islands. The Semi-Arid Tropical Alpine greenhouse accommodates plants growing in tropical high mountain regions. It is unique in its climatic features and the plants growing in it such as the giant Ethiopian Lobelia (*Lobelia rhynchopetalum*). Some very important factors inside this greenhouse are: (i) an alternating day/night rhythm and the lowering of the night temperature down to the freezing point throughout the entire year, and (ii) a high light intensity with high fractions of UV and red light. The Humid Tropical greenhouse is rich in many types of trees, lianas and epiphytes. Bananas (Musa) and Cacao (Theobroma) are native to the tropics and are fruiting in this greenhouse.

Another two experimental-climates investigated in climate chamber are termed: Humid Sub-Arctic (= cool climate) and Arid Sub-Tropical (= dry climate). In general, Arid Sub-Tropical region is warmer during the day, cooler at night and drier during the whole year. The average daytime temperature of Sahara (the largest hot desert of Arid Sub-Tropical region) is ranges from 35° to 42°C, while at night the average temperature goes below 10°C (source: https://en.wikipedia.org/wiki/Sahara, date: 18/11/2014). Therefore, we manipulated the Arid Sub-Tropical experimental-climates in a climate chamber having day/night temperatures around 36/9 °C and mean humidity around 48%. On the other hand, the climate in Humid Sub-Arctic biomes is cool and humid. During the mid-growing season in July, average maximum temperatures of New Siberian Islands (a place situated in the Humid Sub-Arctic biomes) ranges from 8° to 11°C and average minimum temperatures ranges from -3°C to 1°C (Source: http://en.wikipedia.org/wiki/New_Siberian_Islands, date: 18/11/2014). We manipulated the Humid Sub-Arctic climatic conditions also in a climate chamber having day/night temperatures around 11/-5 °C and mean humidity around 67%.

Temperature and humidity of all experimental setups were logged every 10 minutes with the use of HOBO pro v2 onset data loggers (Figure S2). Mean day/night temperature was cool in Humid Sub-Arctic climate (5 °C), moderately cool in the Semi-Arid Tropical-Alpine (12 °C) and Humid Sub-Tropical (18.6 °C) climate, warm in the Semi-Arid Mediterranean (24.1 °C), Semi-Arid Sub-Tropical (24 °C), Humid Tropical (26.4 °C), and Arid Sub-tropical (26 °C) setup. Mean humidity of Arid Sub-tropical climate was extremely low (mean < 50%) than the other experimental setups (mean > 60%; except Semi-Arid Mediterranean = 58.4%, Figure S2). The high differences in temperature and humidity among the target climates created different drying conditions inside the petri dishes even though they were watered similarly in all seven experimental-climates e.g. we visually observed that petri dishes drying out faster in Arid Sub-tropical climates compared to other target climates.

Table 1: Geographic and climatic characteristics of six native and six non-native populations of *V. thapsus* (mean values of native versus non-native ranges are marked in bold). Climatic data were retrieved from the Worldclim database to a resolution of 0.5°, reference period 1959-2000 (Hijmans et al., 2005).

Origin of populations	Population Code	Region of populations	Latitude	Longitude	Elevation a.s.l (m)	Annual mean temperature (°C)	Annual precipitation (mm)
Native range					1587.2	8.6	960.7
Europe	CH5	Grischuns, Switzerland	46.679780	10.164628	1843	2.4	935
Europe	CH2	Haldenstein, Switzerland	46.869583	09.492533	1360	4.4	1211
Europe	CH3	Grischuns, Switzerland	46.700110	08.861860	1100	6.6	1353
Asia	KA3	Kashmir, India	34.250000	74.933330	1940	12.1	795
Asia	KA2	Kashmir, India	34.266670	74.816670	1680	12.3	782
Asia	KA1	Kashmir, India	34.216670	74.783330	1600	13.7	688
Non-native range					760.3	10.5	672.8
North America	MT2	Joebrown, Montana	45.168000	-110.851033	1547	6.5	388
North America	OR3	Wallowa, Oregon	45.282560	-117.769100	1079	6.5	478
North America	OR2	Wallowa, Oregon	45.409170	-117.891800	916	8.1	516
Australia	AU6	New South Wales	-35.316670	149.398600	820	11.6	756
Australia	AU3	New South Wales	-36.563920	149.795400	160	14.9	947
Australia	AU1	New South Wales	-36.539750	149.825300	40	15.3	952

Responses parameters

After the experimental setup, we observed the first seed germination on the 3rd day of the experiment and started data collection thereafter on each alternate day during the first two weeks, and then once a week during the next four weeks. At each sampling point we recorded the new germination and seedling emergence of the target species in all petri dishes. We stopped collecting data after 42 days when we observed no new germination and seedling emergence during the last 7 days. Then we visually checked the viability of seeds that did not germinate. We considered the seeds viable if they did not show sign of fungal attack or rot, and if they were firm when squeezed with tweezers (Seipel et al., 2015). Finally, we added the total number and calculated the relative germination and seedling emergence rates of all viable seeds in percent. We defined "germination" when a seed started emerging its radicle. The appearance of the full radicle marks the end of "germination stage" and the beginning of the "seedling emergence" stage. We defined "seedling emergence" stage when the first leaves called plumule started unfolding, which formed the initial shoots.

Statistical analysis

Linear mixed model (2-factorial) combined with analysis of variance (ANOVA) were used to test the main and interaction effects of experimental-climates and species. Germination strongly correlated ($r^2 = 0.99$) with seedling emergence rates (Figure S3), therefore no separate analysis is shown. We had one non-native population each from *L. polyphyllus* and *S. inaequidens* (both from Europe). For species comparison we, therefore, used the seedling emergence rates of non-native populations of *V. thapsus* from Australia (among the two non-native continents Australia and North America, we selected Australian populations as this is the most distant non-native continent from their native range). Additionally, responses of *V. thapsus* populations were also tested for seedling emergence rates under seven experimental-climate conditions. Linear Mixed-Effects Models (LMEMs) combined with ANOVA were applied to test the significant differences. The model was "response ~ experimental-climates * range of seed region (native or non-native) * mean annual temperature of seed region * annual precipitation of seed region". We used population as a random factor in this model. Regression analysis for seedling emergence rates of *V. thapsus* populations and their climate of seed region were conducted (if the main or at least one interaction effect was significant) with linear least-squares regression (function 'lm').

Homogeneous groups of factor combinations (e.g. experimental-climates * species and experimental-climates * ranges) were identified by Tukey's HSD post hoc comparisons. Prior to statistical analysis, residuals were checked for their homogeneity of variances and also for their normality. All characteristics were tested by examining the residuals versus fitted plots and the normal qq-plots of the linear models (Faraway, 2005). The level of significance was set to $p < 0.05$. LMEMs were conducted with the function 'lme' (Bates et al., 2014) and Tukey HSD post hoc comparisons by the function 'glht' (Bretz et al., 2010). Three packages multcomp (Simultaneous Inference in General Parametric Models, version:1.3-2), lme4 (Linear mixed-effects models using Eigen and S4, version: 1.0-6) and sciplot (Scientific Graphing Functions for Factorial Designs, version: 1.1-0) were used for analysis and graph preparation in R version 3.0.2 (R Core Team, 2013).

Plasticity index ranging from zero to one was calculated for seedling emergence success of three species and also populations of *V. thapsus*. Calculation was done according to Valladares *et al.* (2000): Plasticity index = (Mean maximum seedling emergence among the experimental-climates - Mean minimum seedling emergence among the experimental-climates)/ Mean maximum seedling emergence among the experimental-climates. Means were calculated from responses under five out of seven experimental-climates (as no germination and seedling emergence were observed in other two experimental-climates).

Preparation of germination niche map of three global invaders

We developed the global germination niche map based on climatic conditions under which three global invaders can germinate and emerged. During the germination trial in petri dishes we measured air temperature and humidity of each experimental setup. As germination and seedling emergence were observed at mean temperature above 5°C, we first selected areas in global map based on this condition. Precipitation and length of growing season are two other important abiotic factors for germination and seedling emergence in natural conditions. We did not have precipitation data, and therefore we followed the growing season criterion that is mean monthly amount of precipitation is at least twice as much as the mean monthly temperature. The main goal of germination niche map preparation was to mark suitable climatic region in the global map, where three global invaders can germinate and may establish in the latter life stage. We therefore, used four months growing seasons as three global invaders need this minimum time to germinate, establish and produce viable seeds (Elliott et al., 2011; Ernst, 1998; Gross and Werner, 1978) for next year natural germination and seedling emergence. Then we marked those areas in the global map where above mentioned temperature and precipitation conditions exist at least for four months. Mean monthly temperature and precipitation data were retrieved from the Worldclim database to a resolution of 2.5 arc-minutes, reference period 1959-2000 (Hijmans et al., 2005). Finally, we crosschecked the global germination niche map to see whether it includes all areas of current distribution of three global invaders. Afterwards species-specific niche maps for germination of three global invaders were prepared by marking their respective native range, non-native range (country wise, within the projected germination niche) and the rest were indicated as future niche.

Results

Performances of three global plant invaders under seven experimental-climates

The experimental-climate conditions had significant effects on the seedling emergence rates of three species (Experimental-climate, *F* = 361.8, *P* = <0.0001; Table 2). No germination and seedling emergence occurred under temperature ≤ 5 °C in Humid Sub-Arctic climate (mean humidity 66.7%) and under humidity ≤ 48.4 % in Arid Sub-tropical climate conditions (mean temperature 26 °C). However, seedling emergence observed under other five experimental-climates were structured by temperature and humidity, gradients being significantly lower in two moderately cool experimental-climates (Semi-Arid Tropical-Alpine, 41.7%; Humid Sub-Tropical climates, 53.5%) compared to three warmer experimental-climates (Humid Tropical, 76.0%; Semi-Arid Sub-Tropical, 78.1%; and Semi-Arid Mediterranean climates, 79.0%).

Species responded differently under five out of seven experimental-climate conditions (Species, *F* = 164.8, *P* = <0.0001; Experimental-climate × Species, *F* = 37.9, *P* = <0.0001; Table 2 and Figure 1). Seedling emergence rate of *V. thapsus* was experimental-climate specific. *L. polyphyllus* and *S. inaequidens* performed consistently, but *L. polyphyllus* had higher seedling emergence than *S. inaequidens* in all of these five experimental-climates. *S. inaequidens* performed equally well compared to *V. thapsus* only in the Semi-Arid Tropical Alpine. However, in the other four experimental-climates (Semi-Arid Mediterranean, Semi-Arid Sub-Tropical, Humid Sub-Tropical and Humid Tropical), *S. inaequidens* showed only about half the seedling emergence rate compared to *V. thapsus*. In comparison to *L. polyphyllus*, *V. thapsus* performed significantly lower in Semi-Arid Tropical Alpine (*P* = <0.0001) and Humid Sub-Tropical climates (*P* = <0.0001), equally in Semi-Arid Mediterranean (*P* = 0.9982) and Humid Tropical climates (*P* = 0.9999), and higher in Semi-Arid Sub-Tropical climate (*P* = 0.0023). Plasticity index for seedling emergence success under five experimental-climate conditions was high in *V. thapsus* (0.73), moderate in *S. inaequidens* (0.43) and lower in *L. polyphyllus* (0.25) (Table 3).

Performance of *V. thapsus* populations under seven experimental-climates

Seedling emergence rates of *V. thapsus* populations from native versus non-native range varied by experimental-climates (Experimental-climate × Range, *F* = 28.9, *P* = <.0001; Table 2). Seedling emergence observed only in five (three warm climates: Semi-Arid Mediterranean, Semi-Arid Sub-Tropical, and Humid Tropical; and two moderately cool climates: Semi-Arid Tropical Alpine, and Humid Sub-Tropical) out of seven experimental-climate conditions. In general, responses were significantly higher for non-native populations in three warm experimental-climates (Semi-Arid Mediterranean, *P* = 0.0011; Semi-Arid Sub-Tropical, *P* < 0.0001; and Humid Tropical, *P* <

0.0001), and lower in two moderately cool experimental-climates (Semi-Arid Tropical Alpine, $P <$ 0.0001; and Humid Sub-Tropical, $P = 0.0001$) than native populations (Figure 2). Within the native range, Asian populations surprisingly performed better in Semi-Arid Tropical Alpine (Asia vs Europe, $P < 0.0001$) and in Humid Sub-Tropical climates (Asia vs Europe, $P < 0.0001$) than European populations. Similarly within the non-native range, Australian populations outperformed the North American populations in Semi-Arid Tropical Alpine (Australia vs North America, $P =$ 0.000; Figure S4) and in Humid Sub-Tropical climates (Australia vs North America, $P < 0.0001$; Figure S4).

Table 2: *F* and *P* values of seedling emergence rates of three species and populations of *V. thapsus*.

Factors	Seedling emergence	
	F	*P*
V. thapsus, *L. polyphyllus*, and *S. inaequidens* from non-native ranges		
Experimental-climate	361.8	<.0001
Species	164.8	<.0001
Experimental-climate × Species	37.9	<.0001
V. thapsus populations from native and non-native ranges		
Experimental-climate (biome)	894.8	<.0001
Range of populations (native or non-native)	1.3	0.3258
Temperature of seed source (Mean annual)	20.0	0.0111
Precipitation of seed source (Annual total)	3.5	0.1366
Experimental-climate × Range	28.9	<.0001
Experimental-climate × Temperature	48.5	<.0001
Range × Temperature	0.1	0.8202
Experimental-climate × Precipitation	5.9	<.0001
Range × Precipitation	0.2	0.6636
Temperature × Precipitation	1.7	0.2630
Experimental-climate × Range × Temperature	4.4	0.0002
Experimental-climate × Range × Precipitation	3.1	0.0062
Experimental-climate × Temperature × Precipitation	2.9	0.0089
Range × Temperature × Precipitation	0.1	0.7467
Experimental-climate × Rang× Temperature × Precipitation	3.8	0.0011

Table 3: Plasticity indexes for seedling emergence success of three global invaders. Calculation was done according to Valldares et al. (2000). See methods for details.

Species or populations	Plasticity index
L. polyphyllus	0.25
S. inaequidens	0.43
V. thapsus	0.73
Populations of V. thapsus	
Native range (mean)	0.63
Asia	0.32
Europe	0.93
Non-native range (mean)	0.84
Australia	0.72
North-America	0.95

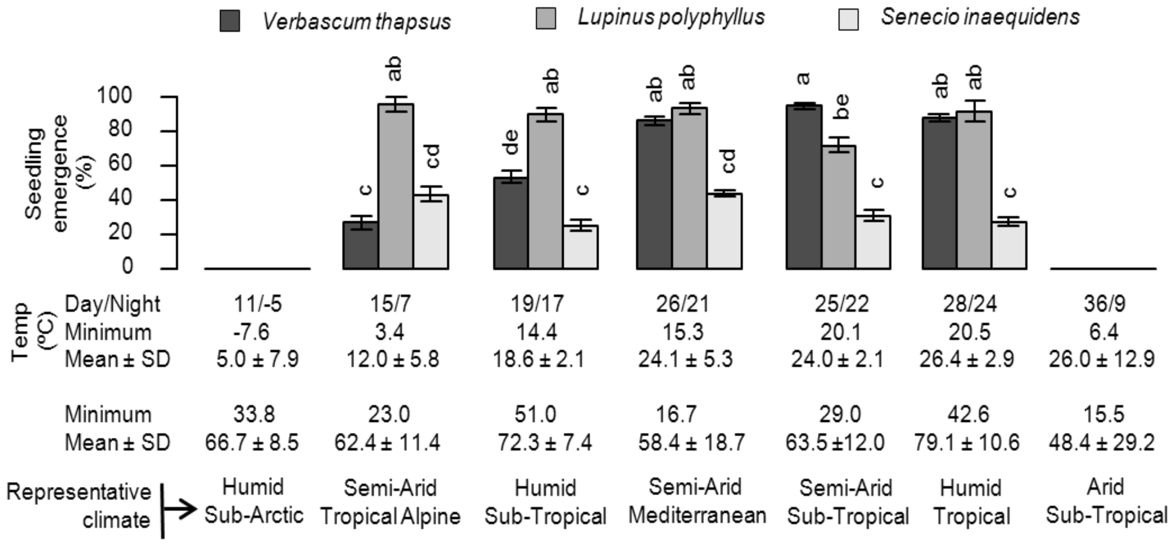


Figure 1: Climatic thresholds to the responses of three global invaders (*V.* thapsus from Australia, *L. polyphyllus* and *S. inaequidens* from German*y*) from non-native ranges. Experimental-climates here are representative to 7 biomes by temperature (^{o}C) and humidity (%). Mean values and standard errors of seedling emergence rates over six weeks are shown here. Significant interaction effect between species and experimental-climates are indicated by different small letters ($P < 0.05$). Note: “Temp” = Temperature; “Humi” = Humidity.

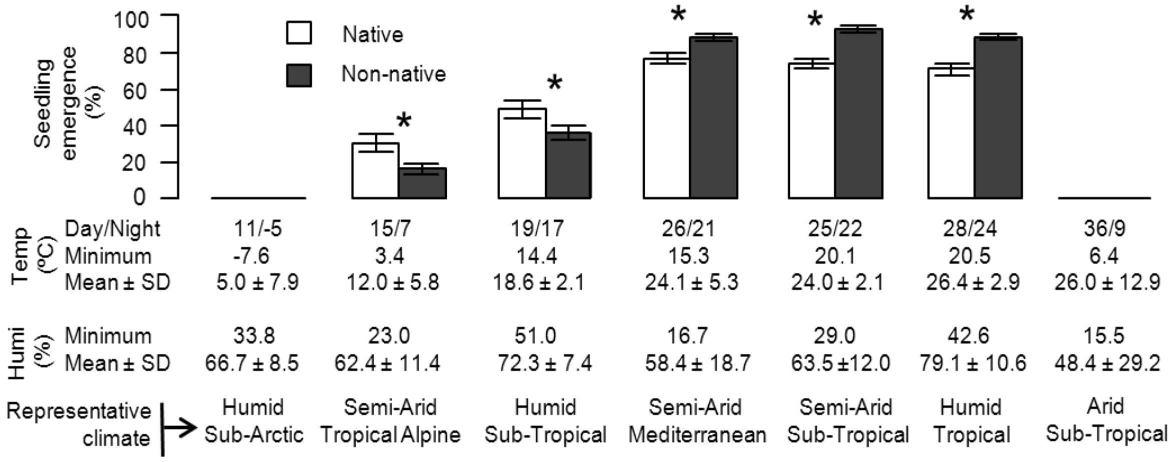
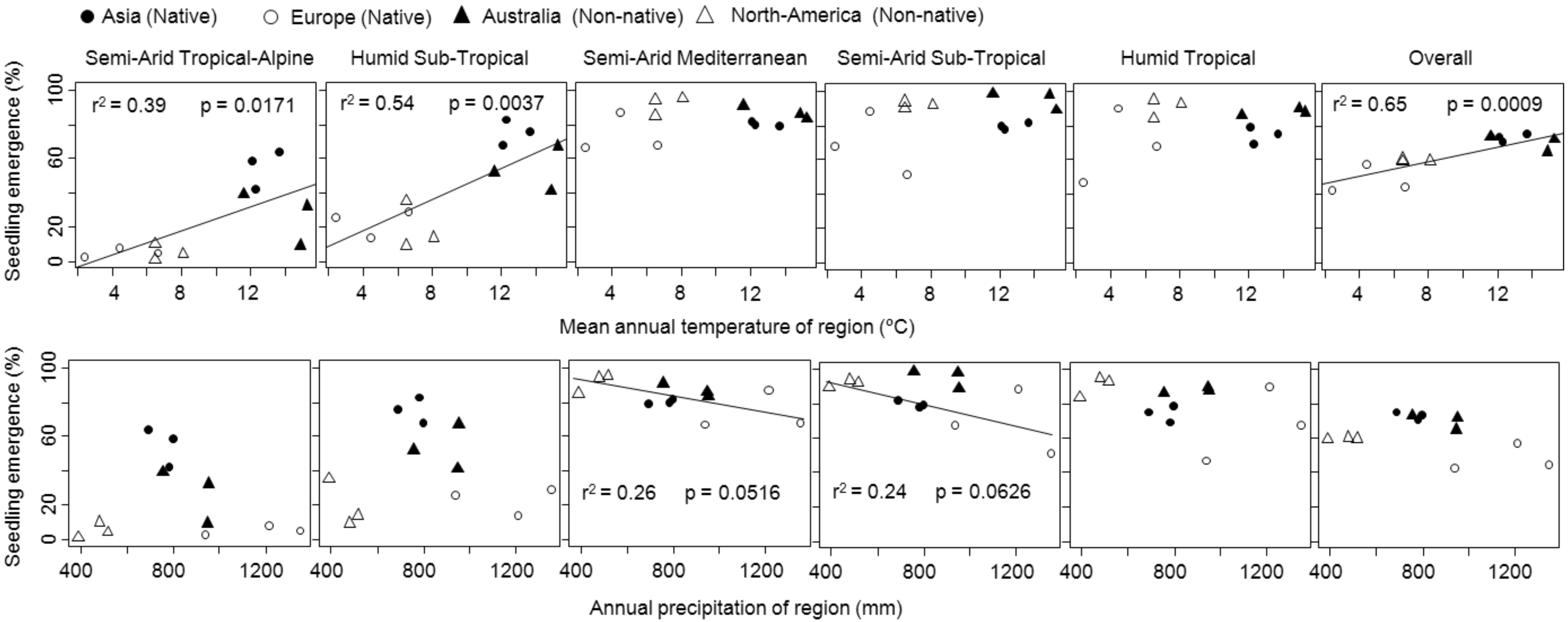


Figure 2: Seedling emergence rates of *V. thapsus* populations under seven experimental-climates. Mean values and standard errors of seedling emergence rates over six weeks are shown in the figure. Significant differences between native vs. non-native populations were indicated by asterisk ($P < 0.05$). Note: “Temp” = Temperature (^{o}C); “Humi” = Humidity (H %).

Figure 3: Relationship between seedling emergence rates and climates of seed region (mean annual temperature and annual precipitation) of *V. thapsus* populations under various experimental-climate conditions (regression lines are shown only for significant, or tends to significant results).



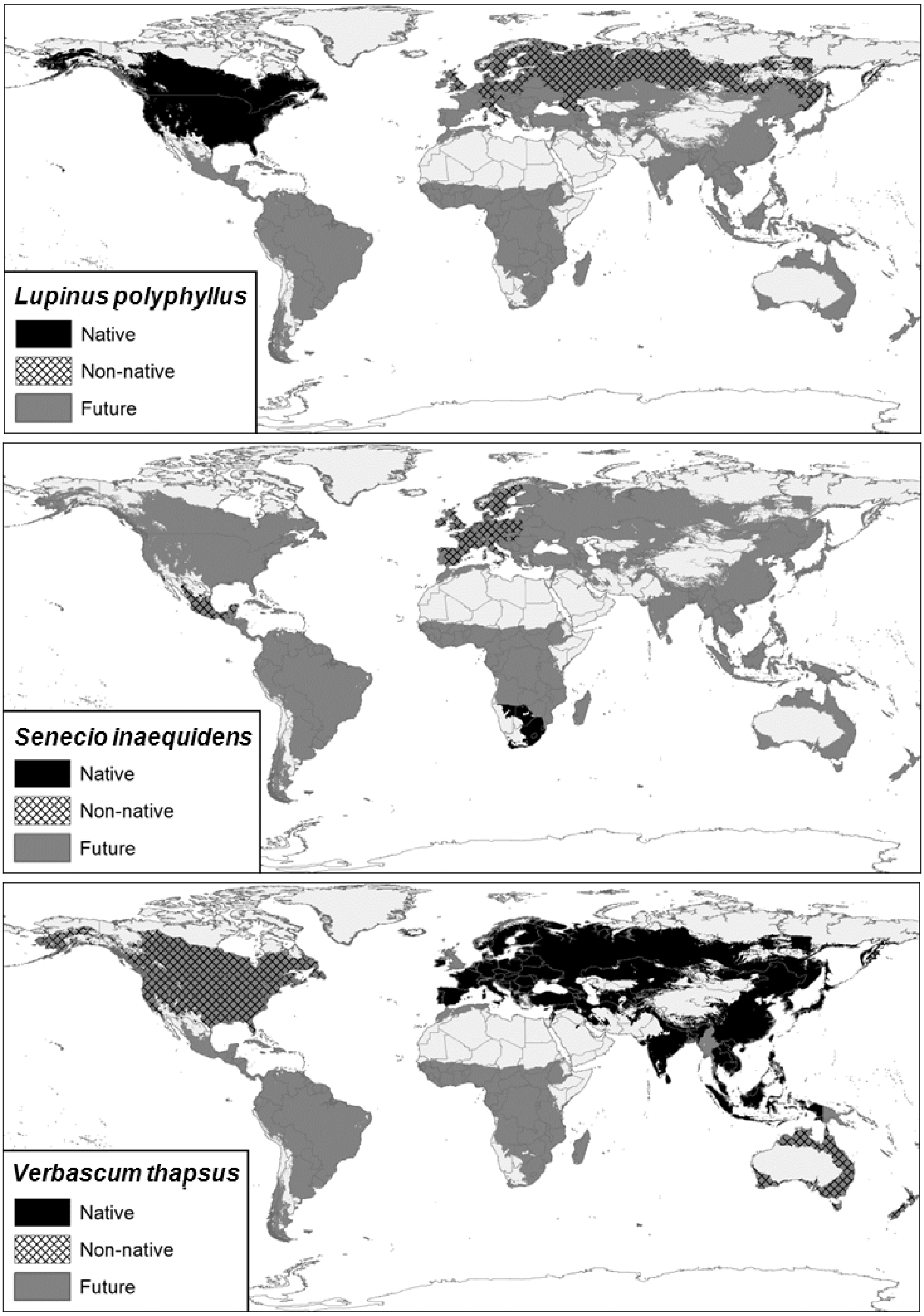


Figure 4: Global germination niche map of three plant invaders: *Lupinus plyphyllus*, *Senecio inaequidens* and *Verbascum thapsus*. Within the germination niche map, native and non-native ranges of three global invaders were marked based on country specific presence absence data (See: Ernst 1998; Sharifnia 2007; Fremstad 2010; Ansari and Daehler 2010; Lachmuth et al. 2010; Seipel et al. 2012; Alba and Hufbauer 2012; Seipel et al. 2015) and rest of the places were marked as future niche suitable for germination success.

Mean annual temperature of seed region had significant positive effects on seedling emergence rates of *V. thapsus* populations in two moderately cool experimental-climate conditions (Semi-Arid Tropical-Alpine, r^2 = 39, *P* = 0.0171; Humid Sub-Tropical, r^2 = 0.54, *P* = 0.0037; Figure 3; Interaction effect: Experimental-climate × range of seed region (native/non-native) × temperature of seed region, *F* = 4.4, *P* = 0.0002; Table 2). No correlation between temperature of seed region and seedling emergence rates of *V. thapsus* populations were observed in other three warmer experimental-climates (Semi-Arid Mediterranean, Semi-Arid Sub-Tropical, and Humid Tropical). Precipitation of seed region had negative effects in two warm and dry experimental-climates with populations from drier region had higher performance than the population from comparatively wet region (Semi-Arid Mediterranean, r^2 = 0.26, *P* = 0.0516; Semi-Arid Sub-Tropical, r^2 = 0.24, *P* = 0.0626; Figure 3**;** Interaction effect: Experimental-climate × range of seed region (native/non-native) × precipitation of seed region, *F* = 3.1, *P* = 0.0062; Table 2).

Plasticity index for seedling emergence success of *V. thapsus* populations differ between native and non-native ranges and also within same range (Table 3). Overall, plasticity index was higher for non-native (0.84) than native populations (0.63). Within the native range, European populations showed higher plasticity (0.93) compared to Asian populations (0.32). Within the non-native range, North-American populations (0.95) had higher plasticity than Australian populations (0.72).

Discussion

Performances of three global plant invaders under seven experimental-climates

We rejected the null hypothesis that seedling emergence of three global invaders will not be hindered by any of the seven experimental-climate conditions, with all three species having consistent performance. Germination and seedling emergence of three species observed in five out of seven experimental-climates suggests that phenotypic plasticity of earlier life traits (here germination and seedling emergence) of three global invaders were limited within an environmental context are in line with the findings of Valladares *et al.* (2007). The potential plasticity in germination and seedling emergence stage might be large but the observed plasticity was lowered due to environmental stress (cool and dry environment here), a findings which is supported by van Kleunen and Fischer (2005). It is also evident that plant species react differently in the extent of their plastic responses to contrasting environmental conditions due to resource limitation (Nicotra et al., 2010; Sultan, 2001; Valladares et al., 2007, 2000; van Kleunen and Fischer, 2005). Phenotypic plasticity of earlier life traits of three target species might influenced by climatic and seed resources in the present study.

Responses of three global invaders were experimental-climate dependent. No germination occurred in Humid Sub-Arctic and in Arid Sub-tropical experimental-climate implying that low temperature or low humidity can limit seedling emergence of three global invaders (*V. thapsus*, *L. polyphyllus*, and *S. inaequidens*). Evidently, an average day/night temperature ≤ 5 °C (night temperatures below -5°C) in Humid Sub-Arctic experimental-climate and an average humidity < 50% in Arid Sub-tropical experimental-climate were the limiting factors for seedling emergence success. In the other five experimental-climates, seedling emergences of all three species were observed indicating species were adapted with those climatic conditions. It is evident that invasive species are able to germinate and establish under a wide range of temperature, for instance, the optimal range for *V. thapsus* is between 20-35 °C (Ansari and Daehler, 2000; Seipel et al., 2015), and the average temperature was within the optimal range in Arid Sub-tropical experimental-climate (26 ± 12.9 °C), but not so in the Humid Sub-Arctic experimental-climate (5 ± 7.9 °C).We watered the petri dishes in all trials, but the low air humidity in Arid Sub-tropical experimental-climate allowed drying out of the petri dishes quicker, potentially exacerbated through high average day time temperatures. We can exclude the possibility that seeds germinated and dried without our knowledge, because we thoroughly examined every seed during the watering of the petri dishes. The average temperature in the Humid Tropical experimental-climate was even higher than in the Arid Sub-tropical experimental-climate and seedling emerged nevertheless due to the higher humidity in Humid Tropical experimental-climate (around 80 %) than the Arid Sub-tropical experimental-climate (below 50 %). Therefore, we recommended that the low air humidity in the Arid Sub-tropical and low temperature in Humid Sub-Arctic experimental-climate inhibited germination and seedling emergence of three global invaders.

Comparing seed traits of three global invaders, one striking difference was the variable seed size. Evidence suggests that seed size affects germination, seedling emergence and early stage establishment (Myint et al., 2010; Townsend, 1992). Among the seeds we used in this study, seeds of *V. thapsus* (0.08 ± 0.03 mg/seed) were comparatively smaller than seeds of *S. inaequidens* (0.21 ± 0.03 mg/seed) and each of them were smaller than seeds of *L. polyphyllus* (24.45 ± 7.97 mg/seed). These differences in seed size could be one explanation of resource-driven germination and seedling emergence success for the *L. polyphyllus* and *S. inaequidens* seeds. *S. inaequidens* exhibited rather low rates in all experimental-climates compared to *L. polyphyllus* implying that the large seeds of *L. polyphyllus* enabled them to germinate and establish in a wide range of different habitats (Sõber and Ramula, 2013). However, the advantage of a larger seed size decreased with more favourable climate conditions, possibly explaining why seedling emergence success of *V. thapsus* getting better with temperature rise even higher than *L. polyphyllus* at least in one experimental-climate condition (Semi-Arid Sub-Tropical climate; temperature, 24°C; humidity, 63.5%).

Phenotypic plasticity can influence performance of invasive species under different climatic conditions in the later life stage (Bossdorf et al., 2005; Nicotra et al., 2010; Sultan, 2001, 2000; Valladares et al., 2007; van Kleunen and Fischer, 2005). The present study showed that phenotypic plasticity of three global invaders differed in their earlier life traits such as germination and seedling emergence success. In addition, different responses of species in five out of seven experimental-climate conditions and different plasticity index suggested species-specific phenotypic plasticity in earlier life traits of three global invaders. Species-specific phenotypic plasticity indicates that three global invaders followed different germination strategies under different experimental-climates: *L. polyphyllus* followed maximum germination strategy across all climates (i.e. low plasticity), *S. inaequidens* followed consistently lower germination across all climates (i.e. moderate plasticity), and *V. thapsus* followed opportunistic germination strategy that higher germination in optimal conditions and lower germination in stress conditions (i.e. high plasticity). The maximum germination strategy of *L. polyphyllus* and opportunistic germination strategy of *V. thapsus* can lead them to establish within a broad geographic range which are in line with the current distribution of target species that *L. polyphyllus* and *V. thapsus* have larger distribution than *S. inaequidens* (Alba and Hufbauer, 2012; Ernst, 1998; Fremstad, 2010; Seipel et al., 2015, 2012; Sharifnia, 2007). Lower germination across all climates might restrict *S. inaequidens* within a small geographic range.

The global germination niche map prepared on the basis of the present study findings suggesting that three global invaders: *L. polyphyllus*, *S. inaequidens*, and *V. thapsus;* are able to germinate and emerge in places outside their current distributions (Figure 4). In general, warm and semi-arid to humid experimental-climates, as tropical, sub-tropical, mediterranean and temperate eco-zones are vulnerable to the crucial first steps of invasion success by these three global invaders. However, seeds experienced a micro-climate condition in the present study, which can be different from prevailing macro-climates in natural conditions. It is evident that macro-climate limits performance of invasive plants at later life stages (Alba and Hufbauer, 2012; Hou et al., 2014). This might explain why the predicted germination niches of three species are larger than the actual distributions of their later life stage, at least in the native range. The Humid Sub-Arctic region is not vulnerable to establishment by three species due to the short growing season (only two months). This two months growing season can provide windows only for germination and seedling emergence (as temperature remains above 5 °C), but no space for flowering and seed production stage as three invaders require around four months growing seasons to reach the seed production stage. In addition, the Arid Sub-Tropic eco-zones are also not vulnerable to establishment by three invader species due to year round low humid weather condition.

Performance of native and non-native population of *V. thapsus* under seven experimental-climates

Our data supported the hypothesis that performances of populations from native and non-native ranges are determined by local adaptation. Indeed, non-native populations stemming from warmer seed region had higher performance in warmer experimental-climate and lower performances in moderately cool experimental-climates compared to native populations and vice versa.

The possible reason why non-native populations performed better only in warmer experimental-climates than native ones and native population performed better only in moderately cool experimental-climates than non-native ones was due to different climatic adaptation of *V. thapsus* populations in their respective seed region. The overall mean annual temperature of the seed region of native populations was significantly colder than the seed region of non-native populations, which provide a hint that native populations were adapted with colder climates and non-native populations were adapted with warmer climates. Seipel et al. (2015) found the same results that a native *V. thapsus* populations (from Kashmir, India) germinated better at low temperatures compared to other non-native populations due to local adaptation.

The present study showed more evidence for local adaptation that the responses of *V. thapsus* populations under different experimental-climates were related to the climate of seed regions (temperature and precipitation of seed source). Positive correlations between temperature of seed origins and responses of *V. thapsus* populations in two moderately cool experimental-climates were due to higher performance of native Asian and non-native Australian populations. These positive correlations did not indicate that populations steaming from warmer region had higher performance in moderately cool experimental-climates rather suggests better performance of populations under least temperature shift (Figure S5). In summary, seedling emergence rate of native and non-native populations depends on the similarities in temperature conditions between climate of seed region and experimental-climates. Eckhart *et al*. (2011) also found that germination of *Clarkia xantiana* populations are related to their temperature of seed region. However, temperature of seed region could not explain the responses of *V. thapsus* populations in other three warmer experimental-climates, possibly because local adaptation cannot influence germination and seedling emergence success under optimal conditions. Moreover, negative correlation (marginally significant) between precipitation of seed regions and seedling emergence rates in two warm and moderately dry experimental-climates indicating that precipitation of seed regions shape responses of populations. Similar findings are presented in a recent study (Hirsch et al., 2012) that precipitation of seed region are negatively correlated with germination response of plant populations. Our data are in accordance with other previous findings (Giménez-Benavides et al., 2007; Kim and Donohue, 2013; Macel et al., 2007; Parker et al., 2003), suggested that seedling recruitment, growth, and reproduction of global invaders in new environments is often determined by local adaptation. The present study showed that local adaptation influences germination and seedling emergence success of *V. thapsus* populations only in moderately stress experimental-climates (moderately cool and dry). In addition, the responses of non-native population are not always higher than native populations. It depends on the weather conditions under which they performs and on the adaptation of population to climate at their respective seed region.

Among the experimental-climates, average high plasticity index of non-native populations than native populations suggesting that seedling emergence success of native and non-native *V. thapsus* populations were also related to phenotypic plasticity. Bossdorf et al. (2005) and Valladares et al. (2007) reported that responses of plant populations under different climate conditions may differ due to phenotypic plasticity. Surprisingly, we found significant differences in seedling emergence rates of both within native and also within non-native populations implying that phenotypic plasticity exist not only between native and non-native ranges but also within the same range. Previous studies regarding performance of native and non-native populations of *V. thapsus* have mainly worked with populations from native European continent or from non-native American continent (Alba and Hufbauer, 2012; Kumschick et al., 2013), except a recent study by Seipel et al. (2015) that investigated performances of populations from Asian (Native) and Australian (Non-native) continents. This could possibly explain why the patterns found here for *V. thapsus* populations are not present in most of the existing studies.

It is also evident that performances of native versus non-native populations in later life stage varied due to genetic variations (Bossdorf et al., 2005; Lachmuth et al., 2010; Maron et al., 2004). Alexander et al. (2012) found genetic clines in response to temperature between native and non-native populations of a global plant invade in their later life stage. Therefore, we cannot exclude the possibility that responses of earlier life stage i.e. germination and seedling emergence success of native versus non-native populations varied in different climatic conditions due to genetic variations. Genetic variation among plant populations within non-native ranges are evident in several previous studies (Meyer and Allen, 1999; Wu et al., 1987), however, not so in populations within native range.

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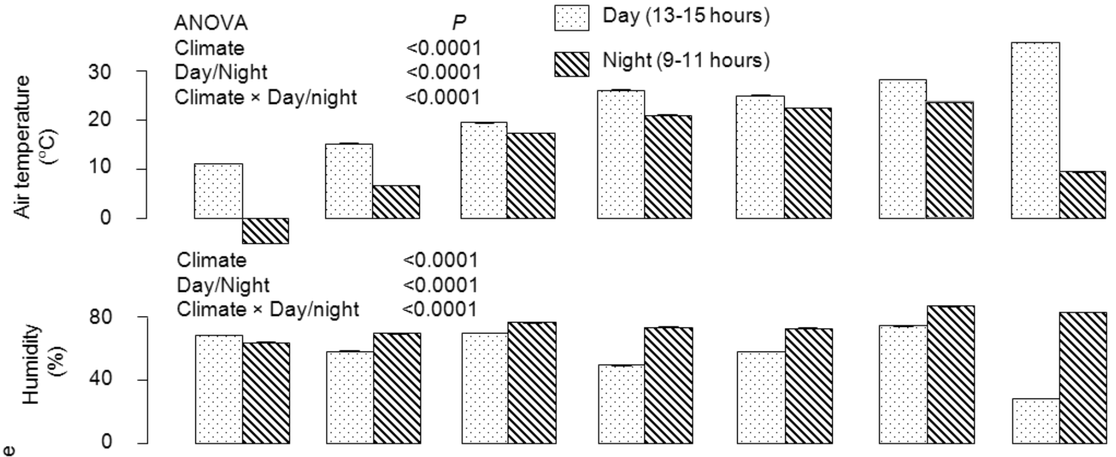
Supporting information

Figure S1: Vegetation inside the five greenhouses of the botanical garden of University of Bayreuth representative to five biomes: (a) Semi-Arid Tropical Alpine, (b) Humid Sub-Tropical, (c) Semi-Arid Mediterranean, (d) Semi-Arid Sub-Tropical, and (e) Humid Tropical (Photos: A. Jentsch).



		Humid Sub-Arctic	Semi-Arid Tropical Alpine	Humid Sub-Tropical	Semi-Arid Mediterranean	Semi-Arid Sub-Tropical	Humid Tropical	Arid Sub-Tropical
Temperature (°C)	Day/Night	11/-5	15/7	19/17	26/21	25/22	28/24	36/9
	Minimum	-7.6	3.4	14.4	15.3	20.1	20.5	6.4
	Mean ± SD	5.0 ± 7.9	12.0 ± 5.8	8.6 ± 2.1	24.1 ± 5.3	24.0 ± 2.1	26.4 ± 2.9	26.0 ± 12.9
	Maximum	14.3	30.9	33.7	41.3	41.9	35.5	39.7
Humidity (%)	Minimum	33.8	23.0	51.0	16.7	29.0	42.6	15.5
	Mean ± SD	66.7 ± 8.5	62.4 ± 11.4	72.3 ± 7.4	58.4 ± 18.7	63.5 ± 12.0	79.1 ± 10.6	48.4 ± 29.2
	Maximum	98.5	99.7	97.6	96.5	94.1	100	99.5
Representative climate →		Humid Sub-Arctic	Semi-Arid Tropical Alpine	Humid Sub-Tropical	Semi-Arid Mediterranean	Semi-Arid Sub-Tropical	Humid Tropical	Arid Sub-Tropical

Figure S2: Day/Night temperature (ºC) and humidity (%) of 7 climate conditions. Mean values and standard errors of per 10 min resolutions (over six weeks) are shown here. Temperature (ºC) and humidity (%) are significantly different between 7 experimental-climate conditions.

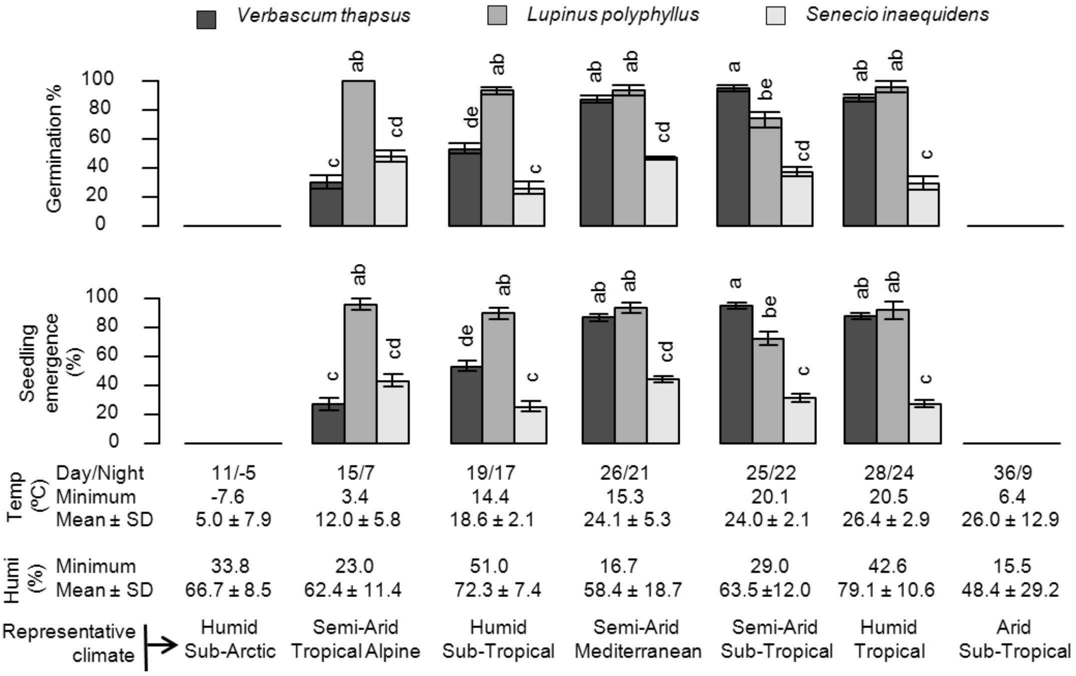


Figure S3: Germination and seedling emergence rates of three global invaders (*V. thapsus*_Australia, *L. polyphyllus*_Germany, *S. inaequidens*_Germany) from non-native continents under seven experimental-climate conditions. Climates here are representative to 7 eco-zones by temperature (ºC) and humidity (%). Mean values and standard errors are shown in the figure. Species responded differently under different climate conditions. Significant interaction effect between species and climates are indicated by different small letters ($p < 0.05$). Note: "Temp" = Temperature; "Humi" = Humidity.

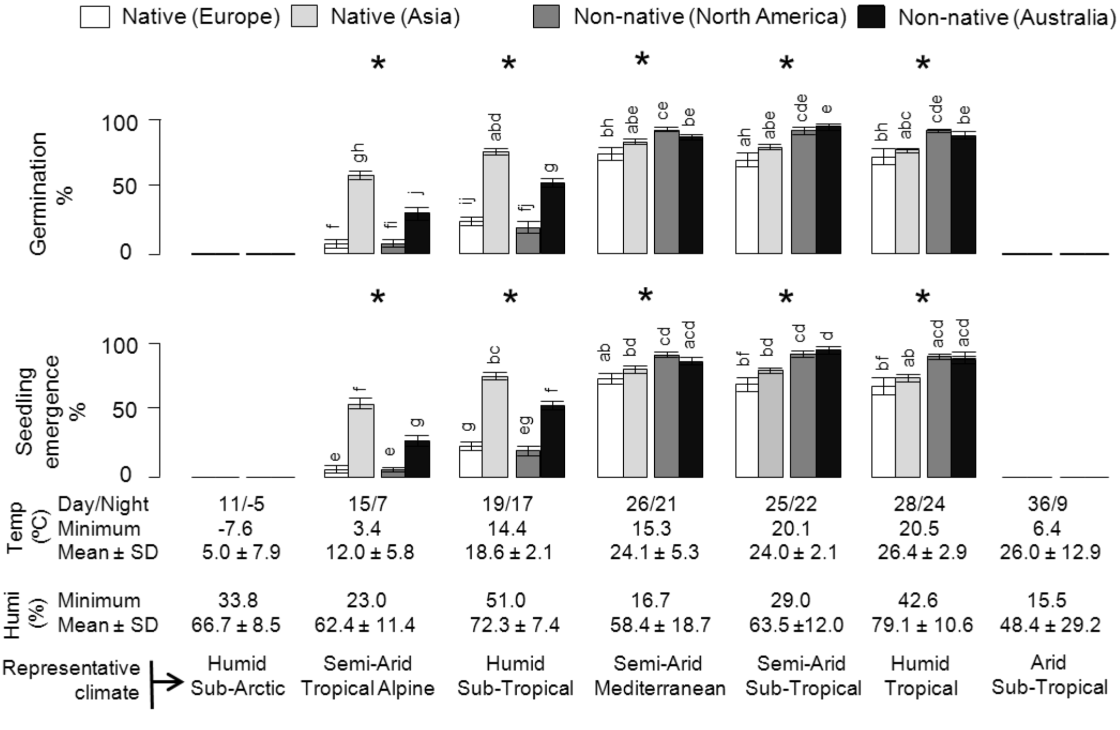


Figure S4: Germination and Seedling emergence rates of *V. thapsus* populations from 4 different continents (2 native and 2 non-native) under 7 experimental-climate conditions. Mean values and standard errors of 3 populations (5 petri dishes each, 20 seeds per petri dish) are shown in the figure. Populations were set as random factor for data analysis. Different small letters indicates the significant ($P < 0.05$) interaction effect between experimental-climates and ranges (native and non-native). Overall significant differences between native vs non-native ranges were indicated by asterisk. Note: “Temp” = Temperature (°C); “Humi” = Humidity (H %).

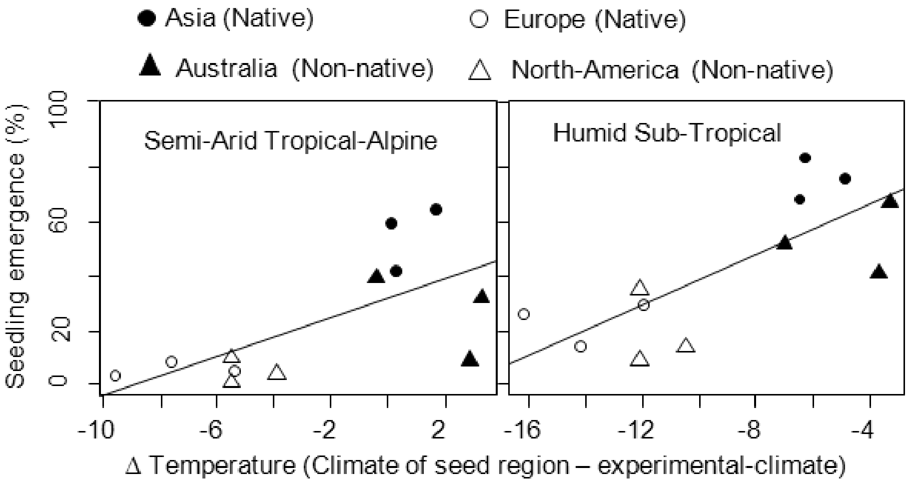


Figure S5: Seedling emergence of *V. thapsus* populations under various temperature shifts (differences in mean temperature of climate of seed region vs. experimental-climate).

6.5 Manuscript 5

Title: Plant responses to climatic extremes: within-species variation equals among-species variation

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Keywords: intra-specific variation, inter-specific variation, extreme climatic events, disturbance, genetic diversity, niche models, provenance.

Abstract

Within-species and among-species differences in growth responses to a changing climate have been well documented, yet the relative magnitude of within-species vs. among-species variation has remained largely unexplored. This missing comparison impedes our ability to make general predictions of biodiversity change and to project future species distributions using models. We present a direct comparison of among- versus within-species variation in response to three of the main stresses anticipated with climate change: drought, warming and frost. Two earlier experiments had experimentally induced (1) summer drought and (2) spring frost for four common European grass species and their ecotypes from across Europe. To supplement existing data, a third experiment was carried out, to compare variation among species from different functional groups to within-species variation. Here we simulated (3) winter warming plus frost for four grasses, two non-leguminous and two leguminous forbs, in addition to eleven European ecotypes of the widespread grass *Arrhenatherum elatius*. For each experiment we measured: (1) C/N ratio and biomass (2) chlorophyll content and biomass and (3) plant greenness, root ^{15}N uptake, and live and dead tissue mass. Using coefficients of variation (CVs) for each experiment and response parameter, a total of 156 within-vs among-species comparisons were conducted, comparing within-species variation in each of 4 species with among-species variation for each seed origin (5 countries). Of the six significant differences, within-species CVs were higher than among-species CVs in four cases. Partitioning of variance within each treatment in two of the three experiments showed that within-species variability (ecotypes) could explain an additional 9% of response variation after accounting for the among-species variation. Our observation that within-species variation was generally as high as among-species variation emphasizes the importance of including both within- and among-species variability in ecological theory (e.g. the insurance hypothesis) and for practical applications (e.g. biodiversity conservation).

Introduction

Ecological theory concerning biodiversity and species coexistence has been based largely on the species concept and has treated species as single, uniform entities across their distribution ranges (Valladares *et al.*, 2014). For example, efforts to describe, preserve and enhance biodiversity are often based on the insurance hypothesis (Walker *et al.*, 1995; Naeem & Li, 1997; Yachi & Loreau, 1999), which is defined as “any long-term effects of biodiversity that contribute to maintain or enhance ecosystem function in the face of environmental fluctuation” (Yachi & Loreau, 1999). Biodiversity insures ecosystem functioning in the context of environmental change or fluctuations; because of differences among species in disturbance tolerance and environmental adaptations, species that are less important or even redundant for ecosystem functioning in one environment might replace others and become key drivers of stability with environmental change (Walker *et al.*, 1999; Fig. 1a, b). The insurance hypothesis is often put forward as an argument for conserving species-rich systems (Yachi & Loreau, 1999). However, within-species genetic and phenotypic variation may be high as well, and at times equal to among-species variation (Hughes *et al.*, 2008; Poirier *et al.*, 2012), potentially being as strong of a buffer for maintaining community stability in the face of environmental change. In support of this statement, higher within-species genetic diversity has been shown to enhance the overall species diversity of a community (Whitlock *et al.*, 2007).

There is a disproportionately low amount of information regarding variation in traits within species relative to among species. There is evidence, however, that variation both within populations (Booth & Grime, 2003) and between populations (Beierkuhnlein *et al.*, 2011; Kreyling *et al.*, 2012) can be important for biodiversity conservation and ecosystem function (Jung *et al.*, 2010). Thus, if within-species differences are as great as among-species differences, the insurance hypothesis could be extended to differentiation within species, and the functional resilience of a community to environmental stress could be ensured through high ecotypic diversity (Fig. 1c, d); ecotypes here mean populations distinguished by morphological and physiological characters, and those that exhibit characters determined by genes restricted to the geographical regions in which they occur (Turrill, 1946). However, high genetic variation within a species is most likely for ecotypes exhibiting high spatial separation. Therefore, assisted gene flow (i.e. the translocation of locally-adapted ecotypes) may be required to significantly increase the stability of an ecosystem in the context of current climate change (Kreyling *et al.*, 2011; Aitken & Whitlock, 2013). A number of strategies (e.g. predictive and composite provenancing) have been developed to optimize vegetation performance using seed sources of different provenances, ranging from simply increasing the genetic diversity within mixtures to determining ecotypes likely to be best adapted to projected climate changes (Breed *et al.*, 2013). As an example of predictive provenancing, using seed sources from the environmental optima of a species in the expanding range limit has been suggested to maximize survival (Atkins & Travis, 2010).

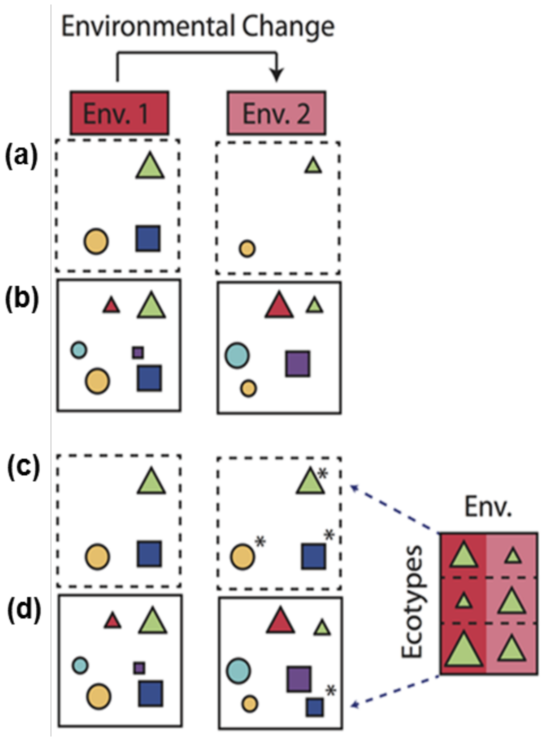

Figure 1: The insurance hypothesis suggests that in species-poor communities (a) functioning is more likely to get lost when compared to species-rich communities (b). In species-rich communities ecosystem functioning can be maintained despite environmental change, because species that are currently functionally redundant and poorly adapted may become important with environmental change (i.e. they might replace other species and take over their role in the system). However, if within-species variability of stress tolerance is as high as among-species variability, negative effects of environmental change may be buffered by (active, human induced or passive natural) introduction or the natural presence of better-adapted ecotypes. The latter is particularly important in species-poor communities (c) and less important in species-rich communities, where other species may maintain ecosystem functioning (d). Colors represent different species, symbols different functions and the size of the symbol the quality of that function within the ecosystem under a particular environment. Asterisks represent newly introduced species.

Adaptation to a local environment in plant species is common, yet the factors that make some species more apt to develop local adaptation are not known (Leimu & Fischer, 2008). Variation in local adaptation could also have important implications for species distribution modelling in response to climate change. Predicting range shifts in response to rapid climate change has become an important topic in ecology, and it commonly results in grim projections with respect to predicted range contractions (Thomas *et al.,* 2004; Thuiller *et al.,* 2005). Most approaches, however, fail to address genetic and phenotypic variation within species. Models of species range limits based on habitat suitability have indicated that incorporation of ecotype-specific responses (i.e. those of locally-adapted populations within species - Hufford & Mazer, 2003) can result in different projections of species range changes as compared with species being treated as uniformly responding units (Oney *et al.,* 2013; Valladares *et al.,* 2014).

An 'extreme climatic event' is an episode or occurrence in which a period of statistical climate extremity alters ecosystem structure and / or function outside the bounds of what is considered typical or normal variability (Smith, 2011). Despite the emerging importance of extreme climatic events as a key component of climate change impacts (Jentsch *et al.,* 2007), empirical data comparing within-species variation in responses to these events to variation among species are lacking. An overview of key recent studies exploring both within- and among-species variation in various measured traits shows that very few studies have explored the relative sizes of both variation types under stress in the same paper (Supporting information S1). Rather, numerous studies have focused on either exploring within-species variation (e.g. Garamszegi & Moller, 2010) and its genetic causes (Ogura & Busch, 2015) or among-species variation (ex., Grime *et al.,* 1997), which shows that both can vary greatly and need to be incorporated in documenting and generalizing plant traits and growth patterns. Increasing climatic variability is expected to increase the frequency of severe heat waves and the frequency and intensity of drought in many regions (Schär *et al.,* 2004; IPCC, 2013), and drought sensitivity is predicted to both change the competitive abilities of plant species and have important impacts at the ecosystem level (Jentsch *et al.,* 2011; Abeli *et al.,* 2014). In addition, an earlier onset of the growing season due to climate change may increase the risk of late frost damage in spring, despite a general air warming trend; this increased risk is expected to occur because the timing of late frost is expected to remain relatively stable (Augspurger, 2013), and the intensity and duration of frost events may not decrease within this century (Kodra *et al.,* 2011). Furthermore, in winter, warm spells can trigger de-acclimation of cold-acclimated plants within hours of warming, leaving plants susceptible to frost damage when freezing temperatures return (Kalberer *et al.,* 2006; Bokhorst *et al.,* 2009). Similar to drought, frost stress can play an important role in influencing plant community composition (Joseph & Henry, 2008), species distributions (Sakai & Weiser, 1973) and overall species diversity (Hettwer *et al.,* 2012). Comparisons of within- vs. among-species variation in responses to warming, drought and frost (the latter in either winter or early spring) are therefore relevant in the context of both community and individual plant stress responses to climate change, and they encompass most temperature related stresses faced by plants.

Whether due to plastic responses, evolutionary change or both, there is mounting evidence for local adaptation in plants (Franks *et al.*, 2014). These local adaptations may even be preserved in the presence of high within-species gene flow (Fitzpatrick et al., 2015). Common European grass species express local adaptations to their climates of origin (Kreyling *et al.,* 2012). Genetic differences among populations within a species have been detected for *Arrhenatherum elatius*, which is a wide-spread and abundant grass species in Europe (Michalski *et al.,* 2010). Here, we compared variation in stress tolerance among and within species by analyzing data from two previous experiments which had exposed ecotypes of four common European grass species stemming from five European countries to simulated summer drought as well as spring frost. We then conducted a winter warming plus frost experiment on four grasses, two non-leguminous forbs, two leguminous forbs and 11 ecotypes of the grass *Arrhenatherum elatius* from different European countries (Ireland, Spain, Germany and Poland) to analyse how variation in responses among species from different functional groups sharing a common origin compare to within-species variation across Europe. We combined data from different experiments (across several years) and multiple stresses (drought, spring frost, winter warming plus frost), several species and also several response parameters in order to obtain results that are as general as possible rather than specific to single settings and species. Based on evidence for occurrences of both high among- and within-species variation to stress in different studies (Supporting information S1), we hypothesized that among species variation does not generally exceed within-species variation for the studied species under a range of stresses.

Materials and methods

Among- and within-species variation in stress responses were directly compared in three different experiments which all quantified plant growth performance under stressful conditions. In order to obtain results that were as general as possible, these three experiments focused on different stresses (drought, spring frost, winter warming plus frost), were carried out using different methodologies (field vs. laboratory experiments), and different parameters were measured (biomass, C/N ratio, chlorophyll, plant greenness, ^{15}N uptake, etc.) in different species and ecotypes. Taken together, the results of the different experiments covered a broad range of conditions which all caused stress and allowed for a direct comparison of among- and within-species variation in stress responses. The drought experiment was carried out in 2009 and 2010, and the spring frost experiment was carried out in 2010; both were part of the EVENT common-garden experiments (Supporting information S2) (Jentsch & Beierkuhnlein, 2010). The location was in Bayreuth, Germany, on the property of the Ecological-Botanical Gardens of the University of Bayreuth, in proximity to EVENT 1 and EVENT 2 (49°55′19″ N, 11°34′55″ E). The long-term mean annual temperature for the site was 8.2 °C, and the long-term mean annual precipitation was 724 mm. The winter warming plus frost experiment was carried out in 2011 as a supplement to the other experiments. It broadened the range of the among-species group (including additional plant functional types) and added another stress. For all experiments plants were obtained as seeds and grown under standardized conditions. All plant species used in the experiments are common grassland species with wide distributions across Europe. All species experience similar management intensity and strong temperature gradients along their distribution ranges, which does not place bias on within-species variation for any particular species. Mean climate parameters and the distributions of all species used in all experiments are provided in Table 1 and supporting information S3, respectively. Mean climate values and standard deviations were calculated using bioclimatic variables downloaded from Worldclim (Hijmans *et al.*, 2005), using a resolution of 10 arc-seconds. Adaptation to local climate was assumed and regional climates were inferred from Worldclim data (Hijmans *et al.*, 2005).

Table 1: Biological information (Source: http://www2.ufz.de/biolflor) and mean climate parameter values and their respective standard deviation values from the distribution ranges of all species used in the experiments. *Arrhenatherum elatius*, *Festuca pratensis, Holcus lanatus* and *Alopecurus pratensis* were used in the drought and warming experiment and the spring frost experiment, while the other species and *Arrhenatherum elatius* were used in the winter warming plus frost experiment.

Species	Pollination type	Selfing	Annual mean temperature (°C)	Mean maximum temperature (°C)	Mean minimum temperature (°C)	Annual precipitation (mm)
Alopecurus pratensis	wind	self-incompatible	4.0 ± 4.6	22.6 ± 3.6	-13.0 ± 8.1	639 ± 219
Arrhenatherum elatius	wind	± self-incompatible	8.3 ± 3.5	23.9 ± 3.8	-5.4 ± 5.3	684 ± 206
Dactylis glomerata	wind	± self-incompatible	5.8 ± 4.8	24.2 ± 4.2	-11.0 ± 7.9	614 ± 219
Festuca pratensis	wind	± self-incompatible	3.8 ± 4.1	23.1 ± 2.8	-14.1 ± 8.1	612 ± 209
Geranium pratense	insect	self-compatible	3.0 ± 3.9	23.1 ± 2.4	-15.7 ± 7.9	579 ± 154
Holcus lanatus	wind	± self-incompatible	8.0 ± 4.0	23.7 ± 4.4	-5.8 ± 5.6	698 ± 256
Lotus corniculatus	insect	self-compatible	7.1 ± 4.4	24.0 ± 4.4	-8.2 ± 6.7	661 ± 244
Plantago lanceolata	wind	± self-incompatible	6.0 ± 4.1	24.4 ± 4.0	-10.9 ± 7.5	600 ± 246
Trifolium pratense	insect	self-incompatible	4.3 ± 4.7	23.4 ± 3.7	-13.3 ± 8.4	608 ± 209

Drought experiment (details on experimental design in Beierkuhnlein *et al.* 2011)

Four grasses from Central European managed grasslands were used (*Arrhenatherum elatius* (L.) P. Beauv. ex J. Presl & C. Presl, *Alopecurus pratensis* L., *Festuca pratensis* H., *Holcus lanatus* L.). Besides local ecotypes of these four species from Germany (DE), we selected other European ecotypes of these grasses from environmentally distinct regions (Italy, IT; Hungary, HU; Bulgaria, BG; Sweden, SE; Table 2). For *A. elatius* and *F. pratensis*, ecotypes from all five target regions were available, while for *A. pratensis* and *H. lanatus* there were only four. Therefore, within-species variation included 5 ecotypes for *Arrhenatherum elatius* and *Festuca pratensis* and 4 ecotypes for *Alopecurus pratensis* and *Holcus lanatus*. Among-species variation included four grass species for Bulgaria, Germany and Hungary and three grass species for Italy and Sweden (Table 2). Four treatments were created in a split-plot design from a replicated factorial combination of temperature (warming and control) and precipitation (drought and control). Twenty one plants per ecotype were used in each treatment.

Table 2: Geography and climate of seed sources of species and ecotypes used in the drought and warming, spring frost and winter warming plus frost experiments. In the winter warming plus frost experiment the shading indicates distinct genetic groupings, as documented by Michalski *et al.* (2012), using pairwise genetic distance scores. Genetic diversity of ecotypes was measured by the proportion of polymorphic loci and by the mean pairwise Jaccard dissimilarity among individuals within ecotypes (*J*), based on amplified length polymorphism (AFLP). Responses of the local *A. elatius* ecotype (marked in bold) originating closest to the other local plant species were treated as part of among-species variation.

Species	Origin	Mean maximum temperature of warmest month (°C)	Latitude	Longitude	Elevation (m a.s.l.)	Mean minimum temperature of coldest month (°C)	Annual precipitation (mm)
Species and ecotypes used in drought and warming experiment and in spring frost experiment							
Arrhenatherum elatius	Bulgaria	24.0	42°00'N	24°50'E	1008	-5.5	658
	Hungary	24.7	47°12'N	17°52'E	440	-5.4	621
	Sweden	20.9	59°51'N	17°38'E	20	-7.5	551
	Germany	22.6	49°17'N	09°58'E	460	-3.7	732
	Italy	30.0	44°55'N	09°44'E	110	-1.8	739
Alopecurus pratensis	Bulgaria	24.0	42°26'N	23°35'E	810	-5.8	593
	Hungary	24.7	47°12'N	17°52'E	440	-5.4	621
	Sweden	20.9	60°00'N	15°00'E	350	-9.8	738
	Germany	22.6	49°17'N	09°58'E	460	-3.7	732
Festuca pratensis	Bulgaria	25.5	42°19'N	23°45'E	710	-4.9	585
	Hungary	25.7	47°27'N	18°28'E	270	-4.7	571
	Sweden	20.9	60°00'N	15°00'E	350	-9.8	738
	Germany	22.6	49°17'N	09°58'E	460	-3.7	732
	Italy	20.7	44°33'N	09°27'E	1600	-3.2	981
Holcus lanatus	Bulgaria	27.7	42°31'N	24°48'E	330	-4.5	581
	Hungary	26.7	46°10'N	17°55'E	200	-4.3	675
	Germany	22.6	49°17'N	09°58'E	460	-3.7	732
	Italy	28.8	44°53'N	09°41'E	160	-2.0	758
Species and ecotypes used in winter warming plus frost experiment							
Arrhenatherum elatius	Ireland 1	19.5	52°38'N	8°57'W	12	2.9	1011
	Ireland 2	18.6	52°30'N	8°51'W	42	1.6	1069
	Ireland 3	18.9	52°03'N	8°30'W	25	3.1	1300
(Within-species diversity	Germany 1	21.5	50°36'N	10°41'E	455	-5.2	673
	Germany 2	20.2	51°44'N	10°45'E	470	-4.2	820

category)	Germany 3	23.4	51°53'N	12°01'E	60	-2.2	493
	Poland 1	23.8	50°34'N	21°40'E	490	-7.3	564
	Germany 4	20.1	51°38'N	10°55'E	490	-4.1	791
	Germany 5	21.6	50°32'N	10°47'E	450	-5.0	672
	Spain 1	23.4	43°15'N	07°17'W	600	2.8	1050
	Spain 2	24.2	42°37'N	08°07'W	545	3.2	1321
	Spain 3	23.2	43°14'N	08°00'W	280	4.1	1175
A elatius, *H. lanatus,* *A pratensis,* *G. pratense* *P. lanceolata* *L. corniculatus,* *T. pratense,* (Among-species diversity category)	Germany	17.7	49°10'N	9°34'E	460	-2.5	676

All seeds of the ecotypes were collected in the wild. Seeds from at least five mother plants per origin were combined to form a mixed sample for each ecotype. The target ecotypes were cultivated from seed simultaneously at the branch office of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Poel, Germany, from February 2009 to April 2009. Collections took place at supposedly autochthonous, semi-natural stands. The seedlings were then transported to Bayreuth and individually planted into 4-L plastic pots filled with local forest topsoil. The soil substrate was sandy silt (pH - 7.27, total C - 1.89%, total N - 0.15%, plant-available NH_4^+ - 1.79 mg L^{-1}, plant-available NO_3^- - 22.50 mg L^{-1}). For the first two weeks after planting the seedlings were watered generously with tap water to ensure growth. All individuals were then cut to a height of 7 cm in order to create comparable starting conditions and exposed to ambient precipitation until the start of the experimental treatments on 25 May 2009. The experiment ended in September 2010. The climate manipulations were performed twice, with the drought lasting 16-19 days in 2009, depending on the species-specific tolerance (drought ended when two-thirds of the individuals from one species showed severe senescence; see below for details), and 30 days for all species in 2010. Twenty one replicates per treatment were used in 2009 and 15 from the 21 were used in 2010 (Supporting information S2).

Drought was induced by rain-out shelters constructed of a steel frame (GlasMetall Riemer GmbH, RahdenSielhorst, Germany) and covered with a transparent polyethylene sheet (0.2 mm, SPR5; Hermann Meyer KG, Rellingen, Germany). The lower edges of the rain-out shelters were at a height of 80 cm and the shelters permitted nearly 90% penetration of photosynthetically active radiation. The control precipitation regime simulated the local daily 30-year average precipitation. The application was done under the shelters twice a week with collected rain water. The extreme drought treatment consisted of a period without precipitation. The drought treatment resulted in a dropping of soil moisture below the permanent wilting point of the soil approximately one week after the start of the treatment in both years. Experimental treatments were continued until a significant number of plants reached a defined state of senescence in 2009. The definition was based on a four-stage visual damage quantification key (0–3, where 0 stands for ‘completely undamaged’ and 3 stands for ‘totally dried out and brittle’). By the time two-thirds of the individuals of one species had reached stages 2 or 3, or by the time one-third of the individuals had reached stage 3, the drought was stopped. The drought treatment lasted 16 days for *H. lanatus*, 18 days for *A. pratensis* and *F. pratensis* and 19 days for *A. elatius*. However, a 30 day long drought treatment was used for all species in 2010. The warming treatment was performed continuously throughout the whole experiment. Warming was done passively via wind-shelters and black floor-covers, which increased the average temperature by 1.5 K compared with the temperature control treatment. All treatments were administered below identical shelters; therefore, the relative temperature and water addition differences were quantified for a single sheltered environment. The fourth treatment was a combination of extreme drought and warming. The additional warming increased the drought treatment effect by additionally reducing the soil moisture by approximately 1.5% on average.

Measured parameters for the drought experiment

Above-ground biomass was harvested at the end of June and the end of September in 2009 and 2010. Leaf C and N concentrations were measured in 2009 after the first drought. Nine replicates per ecotype per treatment were randomly selected (out of 21) for the C/N analysis. A sample for each replicate was taken from the above ground dry biomass, including leaves and shoots, because N concentration varies among different plant parts. Selected samples were fine-milled and mixed well for C and N analysis. Samples (3 mg approx.) were analyzed using an elemental analyzer (EA 3000; Euro Vector, Italy). Relative plant C and N concentrations (%) were provided by this analysis.

Spring frost experiment (details on experimental design in Kreyling *et al.* 2012)

Some of the plants that had been used in the 2009 drought experiment (same ecotype stocks and same individuals) were overwintered outside in a sand-bed and under ambient conditions and used the next year in the spring frost experiment. Since the mean response of all plants within an ecotype (with and without drought history) was used, no ecotypes had biased responses (prior treatment of each plant was used as a random factor to account for the variation in the statistical analyses). Three plants were used from each pretreatment (4 treatments from 2009), resulting in 12 replicates per ecotype per treatment in 2010 for the spring frost experiments. From 26 to 27 May 2010, the plants were exposed to a simulated late frost event (based on local climate data) of –5°C (air temperature) for three hours by gradual cooling them inside a cooler truck. The control (non-frozen) plants remained under ambient conditions.

Measured parameters for the spring frost experiment

The temporal pattern of chlorophyll content was monitored weekly over five weeks after the late frost manipulation using a SPAD-502 chlorophyll meter (Konica Minolta Sensing). Measurements were made on 6 replicates per ecotype per treatment (3 stemming from the drought pre-treatment and 3 from the control of the 2009 drought experiment). Four leaves per plant and date were chosen randomly for the SPAD measurements and an average was taken for the analyses. SPAD-readings were calibrated to foliar chlorophyll content for 20 leaves per species. SPAD readings were conducted for these samples and the leaves were then removed and kept at low temperature without freezing for the immediate determination of the fresh weight. Afterwards, samples were frozen and kept at –29 °C until chlorophyll extraction. For the latter, leaves were cooled in liquid nitrogen and ground in a ball mill while frozen. The chlorophyll was dissolved in 1 ml cold methanol for 30 min and shaken every 10 min. Extracts were centrifuged for 5 min at 4°C and 14 000 rpm. The supernatant was stored in a freezer and warmed for 15 min at room temperature before the absorbance was measured at 645 and 663 nm using a spectrophotometer (DU-50, BECKMANN). The chlorophyll content per g fresh weight was calculated according to Porra (1989) as:

$$[\mathrm{chl}]_{\mathrm{fwt}}\left[\frac{\mathrm{mg}}{\mathrm{g}}\right]=\frac{(8.02\times \mathrm{E}_{663\mathrm{nm}}+20.2\ \mathrm{x}\ \mathrm{E}_{645\mathrm{nm}})}{1000}\times\frac{\mathrm{V(MeOH+watercontent)[ml]}}{\mathrm{freshweight[g]}}$$

A linear regression between SPAD and chlorophyll content yielded significant correlations for all four species with r^2 values of 0.88 for *H. lanatus*, 0. 70 for *A. pratensis*, 0.72 for *F. pratensis* and 0.68 for *A. elatius*. Above-ground biomass was harvested on 6 July 2010 as in the drought and warming experiment, dried for 48 hours at 70°C and weighed.

Winter warming plus frost experiment

Ecotypes of *Arrhenatherum elatius* used in the winter warming plus frost experiment were chosen as genetically distinct seed lines (acquired from the seed bank at the Leibniz Institute of Plant Genetics and Crop Plant Research) based on previous genetic analyses which had been carried out on the same seed sources (Michalski *et al*. 2010). Within-species variation was represented by 11 genetically distinct ecotypes of *A. elatius* selected from four European countries (Table 2). For this species, there is evidence of local adaptation in biomass production after spring frost at the continental scale, whereby ecotypes stemming from regions with a higher incidence of spring frost events were more resilient to spring frost damage (Kreyling *et al.,* 2012). Among-species variation was represented by four grasses (*Festuca pratensis, Holcus lanatus, Alopecurus pratensis, Arrhenatherum elatius*), two non-leguminous forbs (*Geranium pratense* L., *Plantago lanceolata* L.) and two leguminous forbs *(Lotus corniculatus* L., *Trifolium pratense* L.), all sharing the same seed origin (see Table 2).

Plants were cultivated from seed from the end of September to the end of November 2011 at the Leibniz Institute of Plant Genetics and Crop Plant Research. Seedlings were then transplanted into plastic pots (5 cm diameter × 7 cm), using seed compost soil (Einheitserde Classic, Germany). NPK (Mg) liquid fertilizer (15+10+15 (+2)) was applied once at a concentration of 1g/L (Hakaphos Blau, COMPO EXPERT, Germany).

During October and November, the plants were grown in a greenhouse, where night and daytime temperatures averaged 6.4 °C and 20.0 °C, respectively. Light was provided with 400-W lamps (approximately 600 µmol m^{-2} $s^{-1)}$, with a 10 h photoperiod. Plants were transferred to climate chambers at the end of November and for two weeks the day and night time temperatures were lowered to 10 °C and 6 °C, respectively, photoperiod was decreased to 9 h, and PAR light intensity was 200 µmol m^{-2} s^{-1}. To complete plant cold acclimation, the photoperiod was lowered to 8 h for one month, with soil surface temperature averaging 0.0 °C (minimum – 6.2 °C; maximum +5.8 °C). Plants were kept at -1.5 °C prior to thaw treatments, which took place 12-23 February 2012.

On 12 February all plants (6 plants per ecotype and species per treatment) were assigned to one of three thaw treatments: 12 h at 4 °C (mild thaw), 2 days at 9 °C (moderate thaw) or 6 days at 9 °C (extended thaw). Here, we focused on the length of thaw on frost tolerance and not on the effect of frost itself (as compared to the other two experiments, where simply the presence or absence of drought and spring frost were the main stress factors). Freeze-thaw events are known for their ecological importance, and the control therefore included the same number of freeze-thaw events as the manipulations, while they differed in length. Regardless, the gradient of thaw durations was sufficient to generate significant differences in within- and among-species growth responses, which was the main objective. Potential changes in frost tolerance due to the respective thaw periods were assessed by quantifying the responses of the plants to a severe frost event. Frost was administered for 24 h right after the warm spell manipulations. Minimum chamber temperatures in the mild, moderate and extended thaw treatments reached -11.9 °C, -8.1 °C and -8.7 °C, respectively, while the respective mean temperatures were -7.2 °C , -5.4 °C, and -6.7 °C. The only appreciably lower minimum temperature was reached in the mild thaw treatment (i.e. the control) compared with the other two treatments (mean temperature was 1.5 -2 °C lower and absolute minimum temperature was 3.2 – 3.7 °C lower than in the other treatments). Even though the mild thaw treatment reached lower minimum temperatures it still resulted in plants having more than double the growth performance than plants which had experienced milder frost, but after the prolonged thaw/warming. After thawing, all plants were repotted (8 cm × 8 cm × 20 cm deep pots) and transferred to a greenhouse. Temperature was increased by 2 °C every 10 d to simulate spring, reaching ~14 °C on 14 March.

Measured parameters for the winter warming plus frost experiment

Above-ground biomass was harvested one month after the frost for a subset of plants (*n* = 6 per ecotype/ species and warm spell treatment), with brown tissue assigned as dead tissue. Material was dried to a constant biomass at 60 °C and weighed. Percent greenness was quantified from digital pictures under standardized light conditions (a portable light-tight box - 20 cm × 20 cm × 60 cm, and artificial lighting) two weeks prior to the destructive harvest. Greenness calculations (Marchand *et al.,* 2004) used a transformation from the RGB-photos to the HSL color space. Threshold values of the HSL-bands for “greenness” were determined with the remote sensing software ENVI 4.7 and ArcGIS 10. Processing and calculation of greenness percentage were performed with ImageMagick version 6.7.6-5.

A second set of plants was used for destructive analysis of root integrity. Root functional integrity was assessed immediately after thawing by measuring ^{15}N uptake (n = 4 per ecotype and species). Plants and soil were first transferred into plastic cups (5 cm diameter × 10 cm deep). Twelve mL of 100 µM $^{15}NH_3{}^{15}NO_3$ solution was injected 1.5 cm deep into the soil in three aliquots, equidistant from the center. After 22 h of incubation at 20 °C, the plants were rinsed free of soil, washed with 50 ml of 5 mM KCl and 0.5 mM $CaCl_{2,}$ then rinsed with 200 ml of deionized water to remove ammonium passively adsorbed in the root cell walls via cation exchange (Epstein *et al.,* 1963). Roots were excised, and roots and shoots were oven dried separately at 60 ˚C for 48 h and fine-milled and analyzed using an elemental analyzer (see above). Leaf N uptake was quantified only for the mild and extended thaw treatments.

Parameters measured for all three experiments

Biomass was chosen as the central measured parameter because it encompasses the total sum of multiple parameter changes, such as nutrient uptake, height, growth rate, changes in physiology, etc., to show a net treatment effect on plant growth performance. Subordinate parameters were selected based on their suitability to assess the specific stress responses: C/N ratio is representative of plant nutrition differences and has been shown to change after drought stress (An *et al.,* 2005; Sardans *et al*., 2008). Chlorophyll content and greenness are parameters that show abrupt change due to a sudden stress such as frost. Post-frost N uptake ability was used as an indication of root integrity.

Statistics

Overall treatment effects

Linear mixed effects models were used to test treatment effects on all plant species and ecotypes with respect to the measured parameters (C/N ratio, biomass, chlorophyll content, percent greenness, green leaf biomass, dead tissue biomass, ^{15}N uptake). For the drought experiment, the model was “response ~ species*drought*warming + origin*drought*warming”, using total yearly accumulated biomass, experimental unit (each treatment was replicated in 3 randomly assigned shelters), replication and year (for biomass only) as random effects. For the spring frost experiment the model was “response ~ species*frost + origin*frost”, with experimental unit, pretreatment (the previous year drought experiment), replication and date of sampling (chlorophyll measurement only) as random effects.

For the winter warming plus frost experiment one model was used for the species*treatment interaction and another for the ecotype*treatment interaction to show that both species and ecotypes had similar interactions with treatments. To investigate the effect of treatment independent of both ecotype and species, an additional model was implemented, where treatment levels comprised the fixed factor while species- and ecotype-identities were inserted as random effects. Homoscedasticity was checked with residual plots, and normality of residuals was tested with normal probability plots (Faraway, 2005). In the winter warming plus frost experiment all data were square root transformed, while coefficients of variation (see below) were log transformed to satisfy the normality assumption.

Within vs among species variation

We compared variation in stress responses within and among species using coefficients of variations (CVs) (Valladares *et al.* 2006). This method was comparable across the three experiments. Mean response values of every single ecotype within every treatment were used to calculate within-species coefficients of variation, and mean response values of every single species within every treatment were used to calculate among-species CV, for every parameter. The CV among individual plants within an ecotype yielded a single value. The mean of these values (one for each ecotype) was statistically compared against the mean of similarly calculated species CVs. In experiments that had more than one species for within-species variation (the drought and spring frost experiments), separate analyses were run for each species, comparing the variation within that one species with variation among other species (in each of the countries of seed origin). The analyses were run separately to compare variation within each species against among-species variation in each of the countries of seed origin and for each measured parameter for every treatment (e.g. spring frost experiment: 4 Species*5 countries*2 parameters*treatments = 80 t-tests). In each origin-specific within- vs. among-species comparison the local ecotype of each species was included in among- species variation to preserve independence of samples. For example, ecotype “Germany 1” of *A. elatius* (Table 2) was included in the among-species group (from Germany) for the winter warming plus frost experiment, because its seed source was closest to the seed sources of the among-species groups. This ensured that the variation in responses specific to *A. elatius* was also accounted for in the analysis of among-species variation, and that independence of samples was maintained in among- vs. within-species variation analyses. Due to there being no warming effects on biomass and minimal warming effects on C/N ratio compared to drought (Supporting information S4 and S5), CV analyses were done for pooled control (C & W) and polled drought (D & DW) treatments in the drought experiment. Here, temperature manipulation was used as a random factor in the CV analyses.

For the drought as well as spring frost experiments variance partitioning was applied to disentangle the explanatory power of treatment from that of within - and among species variability on the respective measured parameters. Explanatory power is quantified using adjusted R^2 as the goodness-of-fit measure. Joint and independent contributions are estimated by conducting multiple linear regressions with all possible sets of explanatory variables (explained in Legendre 2008). In our experiments, variance partitioning was used to assess the independent contribution of ecotypes to variability of the respective measured parameters that goes beyond variability explained by species. Both species and ecotypes (their origin) were used as factors. Because ecotypes are nested in species, species cannot explain additional variability independently from ecotypes. In the midwinter warming plus frost experiment, partitioning of variation was not possible due to ecotypes being present only within one species. The analysis was conducted using R-package vegan version 2.0-10.

All statistical analyses were performed using R version 3.0.1 (R Development Core Team 2013) and additional packages lmerTest version 2.0-3 for fitting mixed models, multcomp version 1.3-1 for post hoc comparisons, and sciplot version 1.1-0 for graphical illustrations. Species distribution maps (S3) were created from map scans (Meusel & Bräutigam, 1992) using ArcGIS version 10.2.2.

Results

Overall treatment effects on measured parameter values

All three extreme event simulations – ‘spring frost’, ‘drought’ and ‘winter warming plus frost’ – affected most measured parameters related to plant performance negatively (Fig. 2a, 3a and 4a). Species- and ecotype-specific responses were inferred from interactions between species and treatments as well as from interactions between ecotypes (supporting information S4) and treatments. The mean differences among origins and their interactions with treatments represent the influence of ecotypic variation in shaping different species to respond similarly under specific climatic conditions. As both “species” and “ecotype” factors and their interactions with treatments were significant for biomass, chlorophyll content, dead tissue biomass and root N uptake (S4), responses were both species and ecotype-specific.

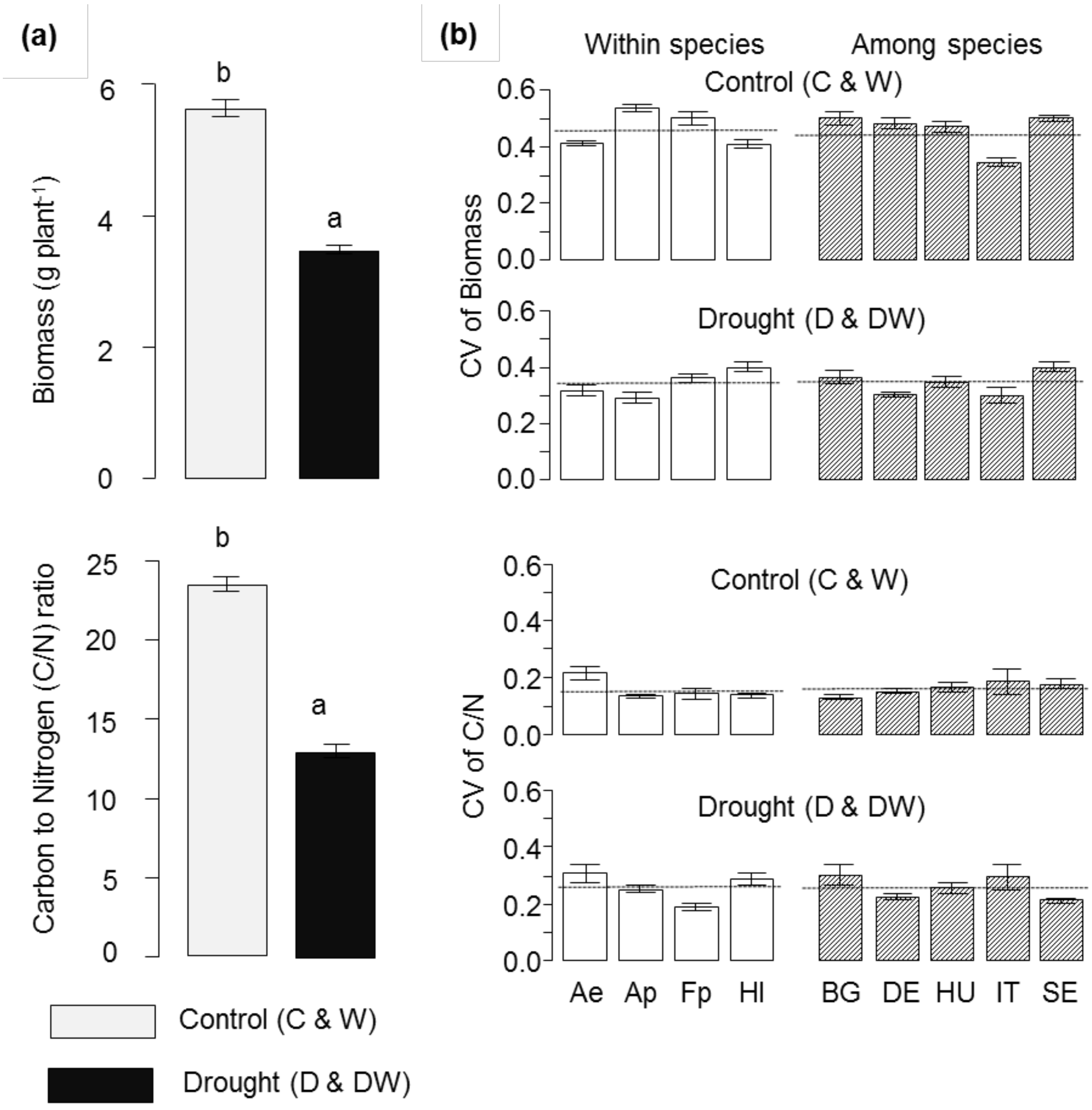

Figure 2: Drought experiment. (a): Overall treatment effects on biomass and carbon to nitrogen ratio using pooled data from all species and ecotypes of the four grass species (see Table 2). Interactions between countries of origin / ecotypes, species and treatments are presented in Supporting information S4a in Appendix. (b): Mean coefficients of variation for each species (4-5 ecotypes per species) represent within-species variation (Ae – *Arrhenatherum elatius*, Ap – *Alopecurus pratensis*, Fp - *Festuca pratensis*, HI – *Holcus lanatus*) while mean coefficients of variation for each origin (3-4 species stemming from each country) represent among-species variation (BG – Bulgaria, DE – Germany, HU – Hungary, IT – Italy, DE – Sweden) for each treatment. Error bars denote standard errors. Different letters indicate significant treatment differences in (a). Dashed lines indicate mean within- and among-species CVs in (b).

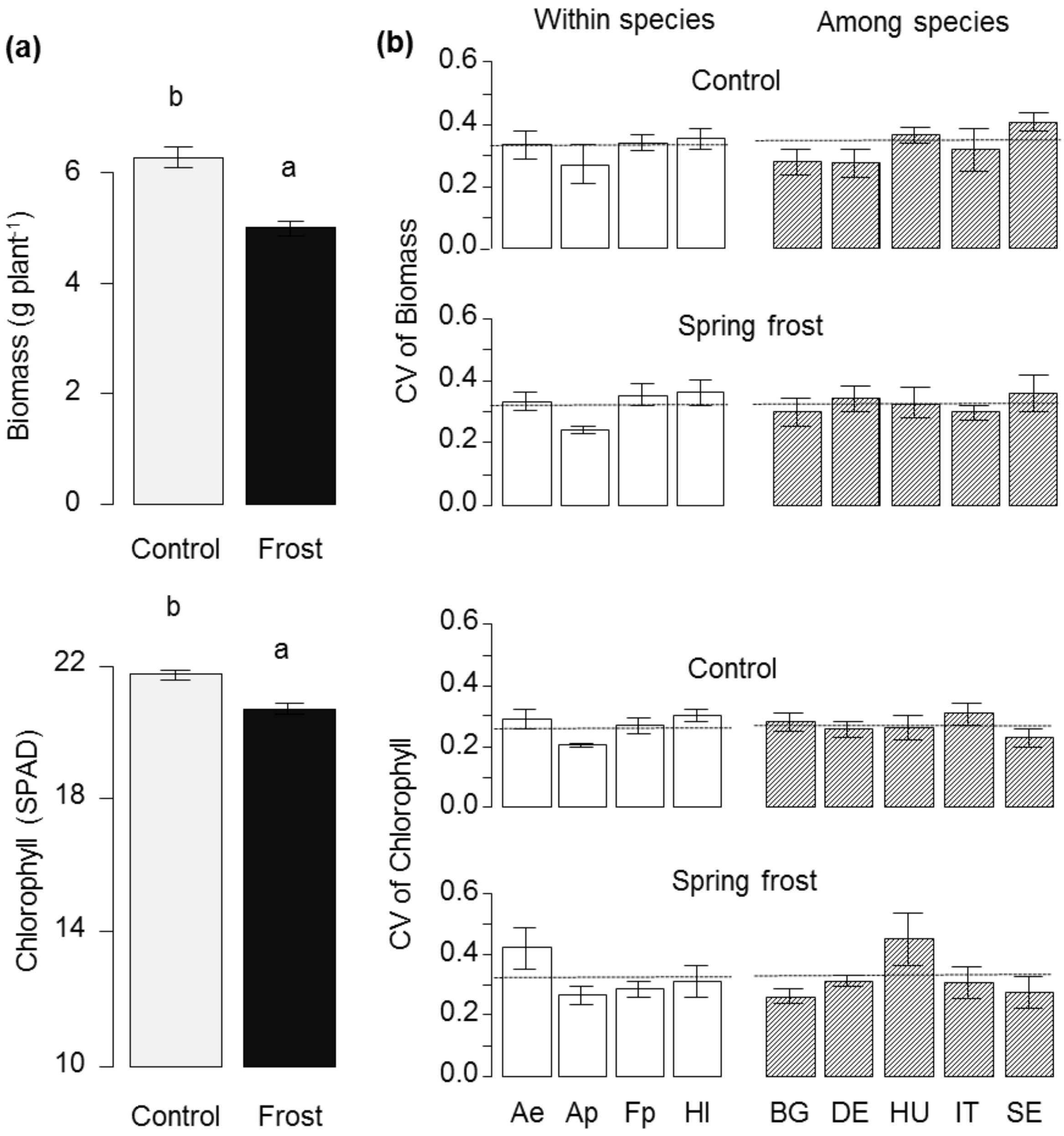

Figure 3: Spring frost experiment. (a): Overall treatment effects on biomass and chlorophyll content using pooled data from all species and ecotypes of the four grass species (see Table 2). Interactions between countries of origin / ecotypes, species and treatments are presented in Supporting information S4b in Appendix. (b): Mean coefficients of variation for each species (4-5 ecotypes per species) represent within-species variation (Ae – *Arrhenatherum elatius*, Ap – *Alopecurus pratensis*, Fp - *Festuca pratensis*, HI – *Holcus lanatus*) while mean coefficients of variation for each origin (3-4 species stemming from each country) represent among-species variation (BG – Bulgaria, DE – Germany, HU – Hungary, IT – Italy, DE – Sweden) for each treatment. Error bars denote standard errors. Different letters indicate significant treatment differences in (a). Dashed lines indicate mean within- and among-species CVs in (b).

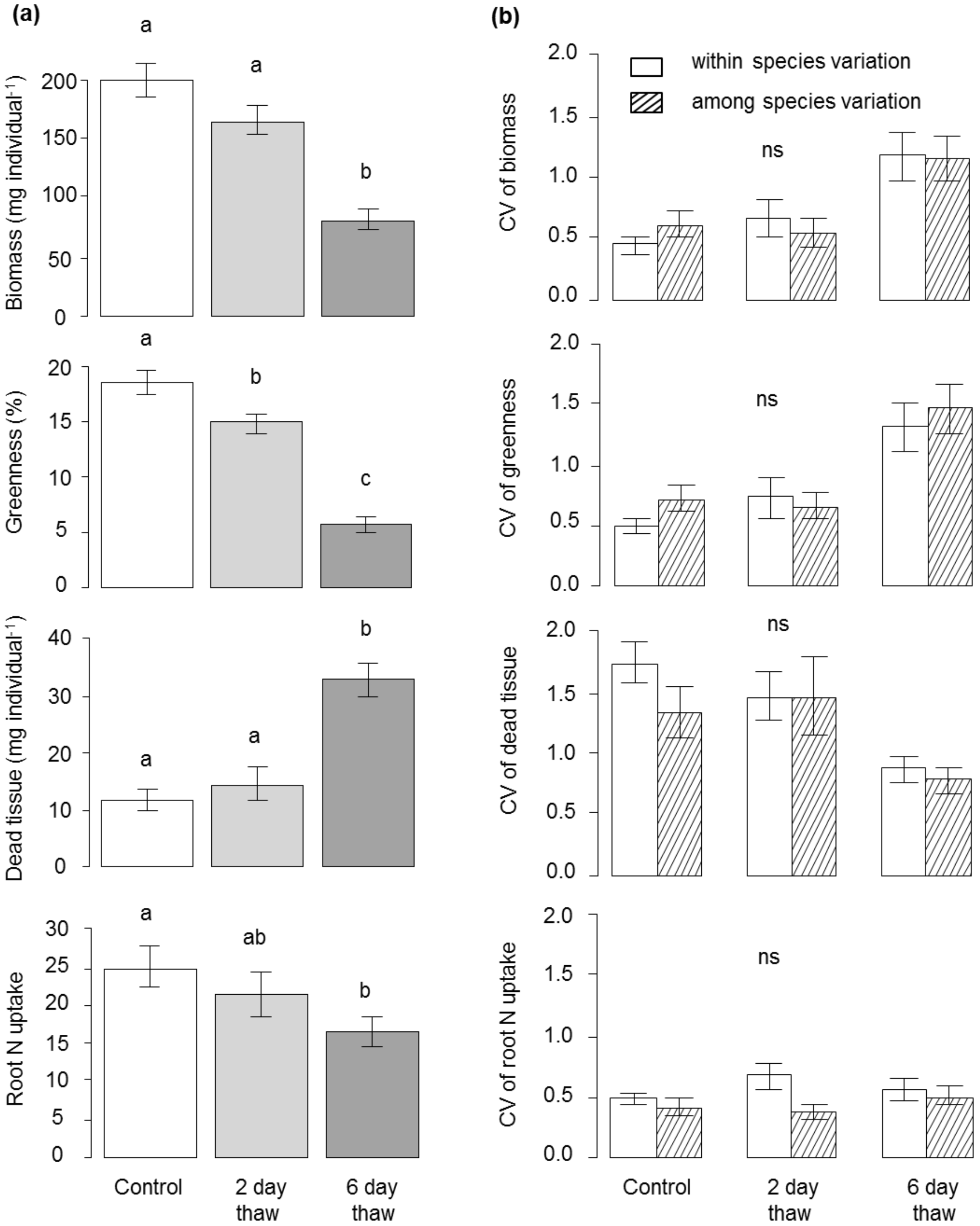

Figure 4: Winter warming plus frost experiment. (a): Overall treatment effects on healthy and dead tissue biomass, greenness and root ^{15}N uptake following a 12 h thaw at 4 °C (Control), a 2 day thaw at 9 °C (2 day thaw treatment) or a 6 day at 9 °C (6 day thaw treatment) using pooled data from all species and ecotypes (see Table 2) Interactions between ecotypes, species and treatments are presented in Supporting information S4c. (b): Mean coefficients of variation for ecotypes vs. species (within- vs. among-species variation) for each parameter in each treatment (11 ecotypes vs. 8 species). Error bars denote standard errors. Different letters indicate significant treatment differences in (a).

Within vs. among-species variation: differences in coefficients of variation

Within-species variation under different extreme events (summer drought and spring frost) matched and at times exceeded among-species variation in four common grass species across five European countries for all tested parameters (biomass, C/N ratio, chlorophyll content in the summer drought experiment as well as in the spring frost experiment, Table 3; Fig. 2b and Fig. 3b). Similar results were obtained when among-species variation in response to winter warming plus frost was expanded to include multiple plant functional groups and additional response parameters (greenness, dead tissue biomass, ^{15}N uptake in the winter warming plus frost experiment, Table 3; Fig. 4b). In total 156 comparisons of within- vs. among-species variation were made, each one representing the within-species variation for a specific species vs. among-species variation in a specific location (origin) for each response parameter and treatment. In six of these comparisons within-species CVs significantly exceeded among-species CVs four times, and was 32% higher on average (see relative effect sizes in Table 3). For the other two significant differences, among-species CVs were on average 40% higher than within-species CVs. For the drought and spring frost experiments, partitioning of variance showed similar among- and within-species explanations of total variation, with a mean of 13% of variation explained by species (and thus ecotypes, which are nested in species), with an additional 9% of variation explained by ecotypes alone, having accounted for species-specific differences (Figure 5).

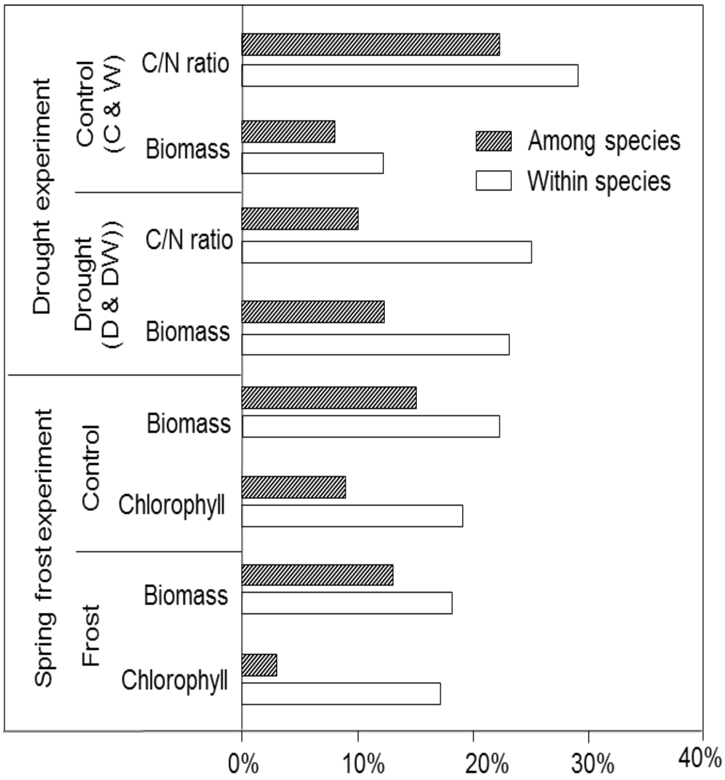

Figure 5: Variation partitioning of drought as well as spring frost experiments, with respect to each measured parameter and treatment. Both species and ecotypes (their origin) are factors , with ecotypes nested in species (one species has several ecotypes). Within-species differences explained an additional mean of 9 % of total variation, after accounting for among – species differences. No additional variation could be explained by species after accounting for the within-species factor as ecotypes are nested within species.

Table 3: Relative effect size (% difference) for within vs. among species coefficients for each treatment in each experiment. Within-species variation in each species (3-4 ecotypes, see Table 2) is compared with among-species variation in each origin (3-4 species, see table 2) for the drought experiment and the spring frost experiment. For each within- vs. among-species comparison, the local ecotype of each species was used as part of the among-species variation. CV = Coefficient of variation. Positive values (% difference) indicate that ecotypic variation is higher than among-species variation, negative values the reverse situation; NA: analysis not done due to unavailable local ecotypes for these species. In (a) treatments were pooled due to the non-significant / low effect size of warming on all plants in comparison with the drought effect. Control and warming treatments were pooled as control (C & W); drought and drought plus warming treatments were pooled as drought (D & DW). In "C" additional species from different plant functional groups were added to among species variation and additional ecotypes were added to within-species variation (see table 2). Significant values (adjusted P < 0.01; according to Bonferroni correction, 4 or 5 multiple comparisons for each response parameter per species per treatment, therefore adjusted P is less than 0.05/4 = 0.013 or 0.05/5 = 0.01) are marked in bold.

		Among species				
Parameter	Within species	Bulgaria (n=4)	Germany (n=4)	Hungary (n=4)	Italy (n=3)	Sweden (n=3)
(a) Drought experiment						
	Control (C & W)					
CV of biomass	*Arrhenatherum elatius* (n=4)	-22	-17	-15	**18**	**-26**
	Alopecurus pratensis (n=3)	3	11	14	NA	7
	Festuca pratensis (n=4)	-3	1	4	**37**	-1
	Holcus lanatus (n=3)	-24	-21	-13	19	NA
CV of C/N ratio	*Arrhenatherum elatius* (n=4)	44	33	23	-7	22
	Alopecurus pratensis (n=3)	9	-12	-31	NA	-40
	Festuca pratensis (n=4)	14	-13	-18	-11	-44
	Holcus lanatus (n=3)	0.4	-13	-12	-34	NA
	Drought (D & DW)					
CV of biomass	*Arrhenatherum elatius* (n=4)	-19	7	-10	13	-37
	Alopecurus pratensis (n=3)	-16	-3	-19	NA	**-53**
	Festuca pratensis (n=4)	-2	19	4	18	-14
	Holcus lanatus (n=3)	2	**30**	11	27	NA
CV of C/N ratio	*Arrhenatherum elatius* (n=4)	-12	32	23	-6	36
	Alopecurus pratensis (n=3)	-18	12	-9	NA	19
	Festuca pratensis (n=4)	-56	-19	-43	-50	-12
	Holcus lanatus (n=3)	-10	28	14	-7	NA
(b) Spring frost experiment						
	Control					
CV of biomass	*Arrhenatherum elatius* (n=4)	19	18	-16	13	-33
	Alopecurus pratensis (n=3)	8	10	-46	NA	-83
	Festuca pratensis (n=4)	16	23	-13	8	-21
	Holcus lanatus (n=3)	25	20	0.1	1	NA
CV of chlorophyll	*Arrhenatherum elatius* (n=4)	-2	14	16	-14	21
	Alopecurus pratensis (n=3)	-37	-25	-25	NA	-17
	Festuca pratensis (n=4)	-9	-1	-1	-12	21
	Holcus lanatus (n=3)	8	19	9	-2	NA
	Spring frost					
CV of biomass	*Arrhenatherum elatius* (n=4)	15	-7	-0.1	15	-13
	Alopecurus pratensis (n=3)	-18	-49	-37	NA	-51
	Festuca pratensis (n=4)	19	8	1	18	-8

	Holcus lanatus (n=3)	13	-2	18	20	NA
CV of chlorophyll	*Arrhenatherum elatius* (n=4)	41	29	-26	29	36
	Alopecurus pratensis (n=3)	0	-21	-81	NA	5
	Festuca pratensis (n=4)	9	-16	-61	1	6
	Holcus lanatus (n=3)	24	2	-70	2	NA
(c) Winter warming plus						
	Control		Germany (n=7)			
CV of biomass	*Arrhenatherum elatius* (n=10)	NA	-43	NA	NA	NA
CV of greenness	*Arrhenatherum elatius* (n=10)	NA	23	NA	NA	NA
CV of dead tissue	*Arrhenatherum elatius* (n=10)	NA	-46	NA	NA	NA
CV of root ^{15}N	*Arrhenatherum elatius* (n=10)	NA	16	NA	NA	NA
	2 day thaw					
CV of biomass	*Arrhenatherum elatius* (n=10)	NA	19	NA	NA	NA
CV of greenness	*Arrhenatherum elatius* (n=10)	NA	-1	NA	NA	NA
CV of dead tissue	*Arrhenatherum elatius* (n=10)	NA	10	NA	NA	NA
CV of root ^{15}N	*Arrhenatherum elatius* (n=10)	NA	**43**	NA	NA	NA
	6 day thaw					
CV of biomass	*Arrhenatherum elatius* (n=10)	NA	1	NA	NA	NA
CV of greenness	*Arrhenatherum elatius* (n=10)	NA	9	NA	NA	NA
CV of dead tissue	*Arrhenatherum elatius* (n=10)	NA	-12	NA	NA	NA
CV of root ^{15}N	*Arrhenatherum elatius* (n=10)	NA	10	NA	NA	NA

Discussion

In our experiments, variation in within-species responses was generally as high as variation in among-species responses under a variety of environmental stressors, and across several species and functional groups. Previous studies comparing within- vs. among- species growth responses have focused primarily on functional trait values along environmental gradients under low or no stress, and in these studies among-species variation has typically been high relative to within-species variation (Albert *et al.,* 2010; Kichenin *et al.,* 2013). Only a few other studies have explored variation in stress responses (e.g. for frost stress, Annicchiarico & Iannucci, 2007, and for drought stress, Poirier *et al.,* 2012), and while they were in agreement with our findings, none of these studies included variation across functional groups to represent among-species variation. Therefore, our study is the first to demonstrate that, at least within common grasses, within-species variation at the continental scale results in ecotypes that react to climate extremes as differently as widely distributed common species (Supporting information S3) from a common origin. This strong influence of within-species variation has immediate theoretical implications within the scope of the insurance hypothesis and practical implications for species distribution modelling and the conservation of biodiversity.

Implications for the insurance hypothesis

Biodiversity encompasses more than species richness, and our results imply that diversity within species may be as important in insuring ecosystem integrity in times of increasing climatic perturbation as species richness. The mechanism behind within-species diversity (presence of diverse ecotypes) enhancing ecosystem integrity is likely to be higher genetic diversity. This may occur via an increase in population fitness, which in turn reduces the risk of species extinction. Higher genetic diversity has been shown to increase the fitness of individual populations by complementary resource use and niche differentiation (Reusch *et al.,* 2005), and via the creation of a stress-resilient portfolio effect, analogous to species rich communities (Schindler *et al.,* 2010). Genetic differences were found within the *A. elatius* ecotypes used in the winter warming plus frost experiment in an earlier study (Michalski *et al*., 2010); however, a definite link between genetic differences among ecotypes and differences in responses was not established in the current study. Phenotypic plasticity is the capacity of a single genotype to produce different phenotypes in response to varying environmental conditions (Whitman & Agrawal, 2009), and it can also be responsible for among- and within-species variation. It was not explicitly quantified in this paper, but it is useful for projecting the ability of ecotypes and species to rapidly adapt to climate change. Most responses to climate change result from phenotypic plasticity and not new adaptations, according to one meta-analysis (Gienapp et al., 2008). Adaptive plasticity can improve survival with environmental change (Chevin & Lande, 2010), thereby compensating for a lack of species and ecotypic diversity, facilitating survival in the context of climate change. High genetic variation within a species is most likely for ecotypes exhibiting high spatial separation, and therefore the dispersal of ecotypes within a species range must also be considered. As mentioned previously, assisted migration of ecotypes may be required to significantly increase the stability of an ecosystem (Kreyling *et al.* 2011). One can either increase the genetic diversity to enhance overall stress tolerance, or if one can accurately forecast the anticipated stress type and magnitude, predictive provenancing may help in selecting provenances which vary less (with lower stress damage compared to present growth values). For a more detailed discussion of this topic we suggest considering the debate on costs and limits of phenotypic plasticity (e.g. Murren *et al.* 2015). The existing potential for maladapted hybrids and outbreeding depression, which can arise after genotypic mixing, can be overcome by following existing guidelines on the population selection process (Whitlock *et al.,* 2013; Frankham *et al.,* 2011). Furthermore, the chance of maladapted hybrids will likely be small in comparison to the negative consequences accompanying species translocations, such as the threat of invasions (Aitken & Whitlock, 2013).

Local adaptation

All grass ecotypes had stemmed from multiple mother plants and were grown in a common environment before being exposed to the different stresses. Therefore, a genetic component to the observed phenotypic variation is likely. The genetic differences among ecotypes were probably due to local adaptation, as shown by the correlations between the climate parameters at the ecotype origins and their treatment responses (Supporting information S6). Due to the presence of only 4 or 5 ecotypes for 2 of the 3 experiments, such correlations are difficult to detect. In the winter warming plus frost experiment stronger correlations were present, although even here it would be more logical to correlate the ecotype responses with the frequency of frosts which occur after warm spells, and those data are not available. As tools to quantify local adaptation we propose (1) establishment of multiple common gardens along latitudinal/altitudinal gradients, (2) in situ additional climate manipulations and (3) manipulations in climate chambers. Detailed methods on these procedures in the context of frost adaptation can be found in Malyshev et al. (2014). Overall, the speed of evolution of local adaptation should always be evaluated to the same degree as species-specific adaptations for theoretical considerations in ecology, such as coexistence theories.

Implications for predicting changes in species distributions

Our result of within-species variation in drought and frost tolerance being as high as among-species variation in different functional groups emphasizes the importance of incorporating within-species variation into projections of climate change responses (Valladares *et al.,* 2014). However, the speed at which ecotypes that are ill-adapted for future climate might be replaced by better adapted ecotypes is an important, yet hardly known, piece of information required for sound projections of species’ responses to climate change. Decision makers responsible for plant transplantations (e.g. foresters, urban landscape planners) and ecotypic seed mixing (e.g farmers) thus need to acquire the necessary information on ecotype performance to make informed decisions.

Micro-evolutionary adaptation to drought can occur within short geographic distances in forest tree species, and such adaptations can easily spread via gene flow (Pluess & Weber, 2012). Alternatively, the assisted colonization of pre-adapted ecotypes of key species within their current range may contribute to the functional integrity of ecosystems, without the need to introduce exotic species with unknown risks (Kreyling *et al.,* 2011). The level of ecotypic variation and ability to evolve new ecotypes within a species are therefore important characteristics to consider when evaluating range shifts of species driven by environmental stressors. Local adaptation has been detected in only 71% of transplant studies (Leimu & Fischer 2008; Hereford, 2009), which could be explained by species-specific differences in the extent of within-species variation under stress. Therefore, our results highlight the importance of identifying factors and species traits responsible for evolving new ecotypes, both of which might play a crucial role in determining the most vulnerable species under climate change.

Ecological implications of drought and frost responses

Drought, spring frost damage and winter warm spells are likely to increase in the future (IPCC, 2012). Drought duration and spring frost magnitude in our experiments were selected based on local climate patterns and projections (see Beierkuhnlein *et al.,* 2011 and Kreyling *et al.,* 2012) and therefore represent realistic scenarios. Likewise, our winter warming plus frost simulation resembles natural winter warming events lasting 5 days or more with temperatures reaching over 5 °C, which have occurred approximately once every seven years between 1913 and 2000, even at locations much colder than our sampled sites (Bokhorst *et al.*, 2008). The latter implies that warm spells of this magnitude and duration are also likely to occur at lower latitudes as well. Field experiments have shown comparable growth reduction after extreme winter warming, as observed in our study, with week-long temperatures in winter of around 7 °C reducing summer growth by 87% in dwarf shrubs (Bokhorst *et al.,* 2009). In this respect, the testing of frost responses after winter warm spells lasting two and six days at 9 °C was realistic in the context of winter climate change.

Considerations regarding interpretation of results

We compared among – and within-species variation only in species from extensively used grasslands which are widespread across Europe, species rich (commonly >20 species per m²), characterized by low management intensity (not ploughed, not sown, unfertilized or subject to low, mainly organic, fertilization), and used as meadows for hay or silage or as pastures (Beierkuhnlein et al. 2011). Still, these grasslands depend on human use (if missing, succession towards forests takes place in a few decades). This specific disturbance regime might have selected for rather similar species. Phenology has been shown as a key predictive factor in explaining tree species distributions (Chuine and Beaubien, 2001; Morin et al., 2007. Among- and within-species differences in phenology with respect to temperature changes were not tested here and are suggested to be compared in future studies. Nonetheless, for a defined set of growth response parameters we have shown that for each of the 4 common grassland species within-species variation was not lower than among-species variation. One promising future research direction is thus to quantify the effect of within-species variability for different plant functional groups.

Species interactions can influence the evolutionary paths of different plant traits (Tilman & Snell-Rood, 2014). Greater trait differentiation develops in species grown in more diverse communities, which promotes stronger selection pressure compared to species grown in isolation (Zuppinger-Dingley *et al.,* 2014) or under anthropogenic influence such as fertilization (Hautier *et al.*, 2014). In this respect, species mixtures may still be more influential in leading to trait diversity of a community compared to ecotype mixtures. Experiments studying evolutionary processes in different ecotype and species mixtures are therefore currently of high importance. Overall, we suggest five research questions to spur further research to explain and make use of our finding of potentially equal variation in growth within and among-species after stress: (1) What is the speed of evolution of local adaptations and plant ecotypes? (2) What happens, with respect to performance and response variability, if geographically isolated ecotypes are mixed by humans

(Fig. 5)? (3) How does within-species variability in stress responses vary among species and what drives this variability (species, functional groups, generation length, range size etc.)? (4) Are certain factors more influential in the development of within-species variability (adaptation to climate, environmental opportunity, space, genetic isolation by geographic isolation)? (5) Are certain environmental thresholds (e.g. minimum temperature) harder to cross by within-species variability in stress tolerance than among species?

In summary, our study explored the relative importance of within- vs. among-species variation in response to multiple stress factors and disturbance interactions – drought, warming, frost and their combinations. In addition, we explored within-species variation in four grass species and among-species variation in multiple functional groups and quantified several response traits. Taken together, we present general evidence that response-variation within single species across their ranges can match the response-variation encompassing different plant functional groups at single sites under stress. This contrasts previous reports, conducted under non-stressful conditions, where among-species trait variation dominates. Within-species variation should therefore be included in the refinement and testing of general ecological theories and ecological applications such as species distribution modeling and biodiversity conservation.

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Supporting information S1: Key recent studies comparing among vs. within-species variation in the same paper, showing the dominant variation source in each study. Whether additional stress was induced in the study through the experimental design is noted under "presence of induced stress". The literature search was done in Web of Science on the 28th of November 2014 using the keywords: (within.species OR intra.species) AND (among.species OR inter.species) AND plant AND variation), which yielded 266 results. All search results were checked for studies where variation in plant traits and / or responses to environmental changes (excluding genetic analyses) was analyzed in the same paper and partitioned into among and within-species variation and subsequently compared.

Type of study	**Species**	**Presence of induced stress**	**Parameters**	**Dominant variation**	**Effect size**	**Reference**
1. Field drought and warming experiment	*Dactylis glomerata*. and *Festuca arundinacea;* two populations per species from mediteraniean and temperate origins.	under stress	biomass, nitrogen nutrition, survival	within-species	"much greater"	Poirier *et al.*, 2012
2. Natural gradient sampling	two tree species (*Larix decidua, Pinus resinosa*); 27 tree populations per species (30 km apart)	without stress	leaf chemichal composition, N resorption, corbon isotope discrimination,SLA, lifespan	within species	2 to 3 times	Walters & Gerlach, 2013
3. Field winter survival experiment	farba bean, field pea and white lupin; 10 or more cultivars per species from southern and western or central Europe	without stress	winter survival	within-species	"much greater"	Annicchiarico & Iannucci, 2007
4. Wood anatomy sampling along wide climatic gradient	139 tropical trees across families and their populations	without stress	wood anatomical properties (eg.,vessel cross-sectional area)	within - species	NA	Fichtler & Worbes, 2012
5. Meta-analysis (observational studies)	various	without stress	functional traits (leaf mass : area, N content)	within - species / equal	NA	Read *et al.*, 2014
1. Glasshouse experiment simulating winter and tropical growth conditions	four oak species and one to five populations per species (US, Mexico, Costa Rica)	under stress	growth rate, freeze tolerance	among - species	2 to 6 times	Koehler *et al.*, 2012
2. Global database	129 alien species	without stress	plant functional traits (height, biomass, SLA)	among - species	NA	Ordonez, 2014
3. Matrix population models from literature data	50 perennial plant species; multiple populations (≥ 2; ≥ 1 km apart) and multiple	without stress	population growth rate	among - species	NA	Buckley *et al.*, 2010

	matrices per population					
4. Field leaf measurements	171 species (grasses, herbs and woody species) in 174 sites across Chinese grasslands, Tibetan Plateau, Inner Mongolia, and Xinjiang.	without stress	leaf traits	among - species	7 times	He *et al.*, 2010
5. Review paper	C3 species	without stress	photosynthetic capacity	among - species	NA	Kouki Hikosaka, 2010
6. Elevational gradient	31 dominant and subordinate species in New Zealand along 900 m; 10 populations per species	without stress	leaf traits (dry matter content, N and P concentrations, area and SLA)	among - species except for SLA.	3 times or more	Kichenin *et al.,* 2013
7. Wood elemental analysis	nine *Shorea* tree species, one to five populations per species	without stress	physical and chemical wood characteristics	among -species	NA	Pande *et al.,* 2007
8.Environmental gradient sampling	13 common plant species	without stress	five functional traits	among -species	~2.5 times	Albert *et al.,* 2010
1. Gradient litter nutrient analysis in six long-term chronosequences	four to six vascular species per chronosequence; two populations per species in Boreal, temperate and subtropical zones	without stress	nutrient concentrations	variable, depending on the chronosequence examined	NA	Wardle *et al.,* 2009
2. Climatic gradient leaf measurements (Tropical cloud forest)	mean of 33 species in each of three forest sites, spanning (1263-1436 m.a.s.l); 10 to 16 populations per forest site per species.	without stress	SLA	approximately equal	NA	Long *et al.,* 2011
4. Geographical gradient	three Cuban *Pinguicula* species; 1 to 4 populations per species (approx. 2 km apart)	without stress	31 morphological quantitative traits	similar among and within-species trait differentiation along environmental gradient	NA	Dominguez *et al.,* 2014

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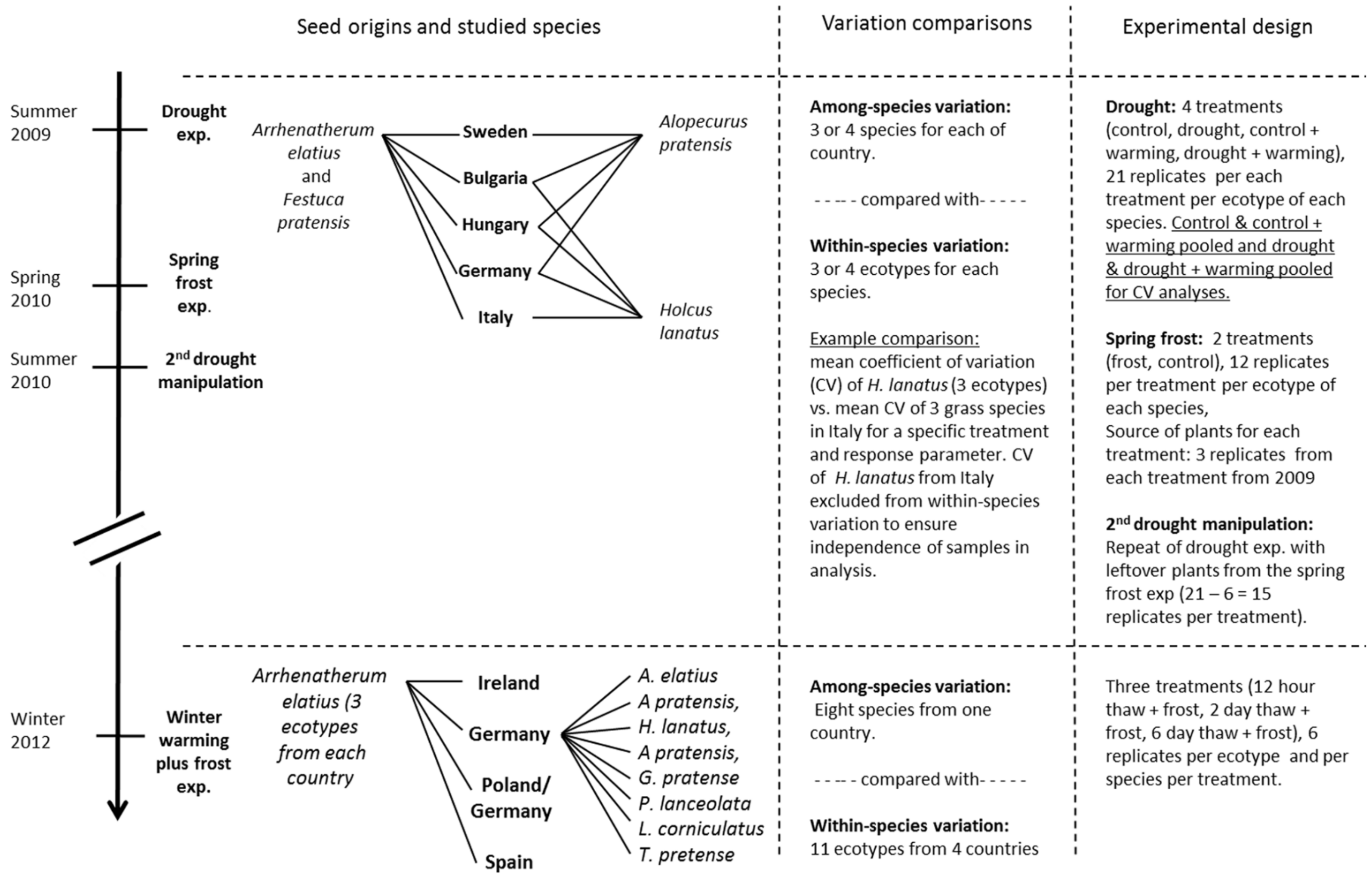
Supporting information S2: Sequence of the three experiments and their respective constituents, making up among- and within-species variation. Countries represent the respective ecotypes of each species (their seed origin). Experimental design describes how each stress type was simulated to generate variation in plant responses.



Among-species variation: 3 or 4 species for each of country.

- - -- - compared with- - - - -

Within-species variation: 3 or 4 ecotypes for each species.

Example comparison: mean coefficient of variation (CV) of *H. lanatus* (3 ecotypes) vs. mean CV of 3 grass species in Italy for a specific treatment and response parameter. CV of *H. lanatus* from Italy excluded from within-species variation to ensure independence of samples in analysis.

Drought: 4 treatments (control, drought, control + warming, drought + warming), 21 replicates per each treatment per ecotype of each species. Control & control + warming pooled and drought & drought + warming pooled for CV analyses.

Spring frost: 2 treatments (frost, control), 12 replicates per treatment per ecotype of each species,
Source of plants for each treatment: 3 replicates from each treatment from 2009

2nd drought manipulation: Repeat of drought exp. with leftover plants from the spring frost exp (21 – 6 = 15 replicates per treatment).

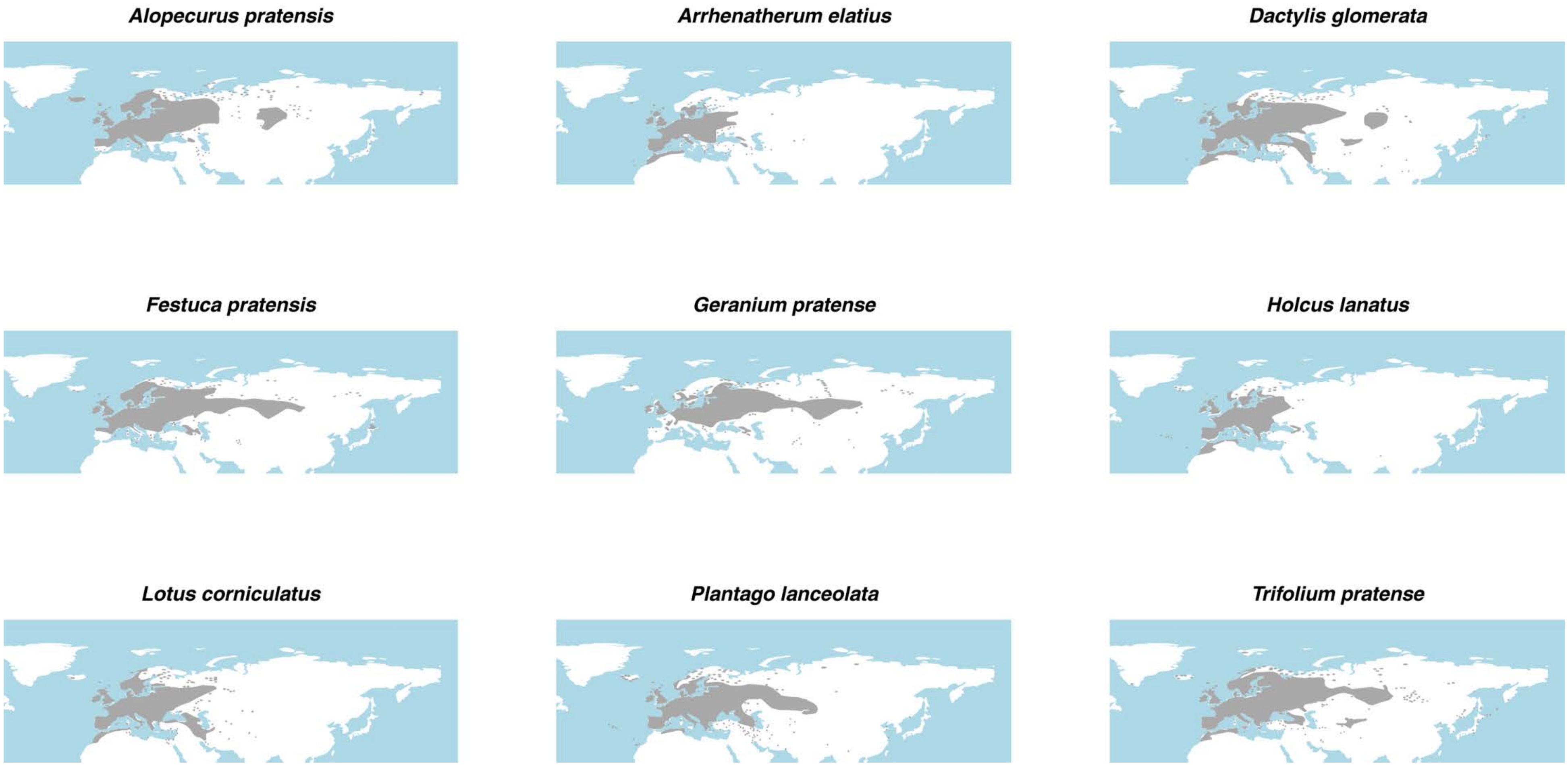
Among-species variation: Eight species from one country.

- - -- - compared with- - - - -

Within-species variation: 11 ecotypes from 4 countries

Three treatments (12 hour thaw + frost, 2 day thaw + frost, 6 day thaw + frost), 6 replicates per ecotype and per species per treatment.

Supporting information S3: Natural distribution ranges of all species used in the experiments. *Arrhenatherum elatius*, *Festuca pratensis*, *Holcus lanatus* and *Alopecurus pratensis* were used in the drought and warming experiment and the spring frost experiment while the other species and *Arrhenatherum elatius* were used in the winter warming plus frost experiment. Images were obtained from scanned species distribution maps (Meusel & Bräutigam 1992).


Alopecurus pratensis

Arrhenatherum elatius

Dactylis glomerata

Festuca pratensis

Geranium pratense

Holcus lanatus

Lotus corniculatus

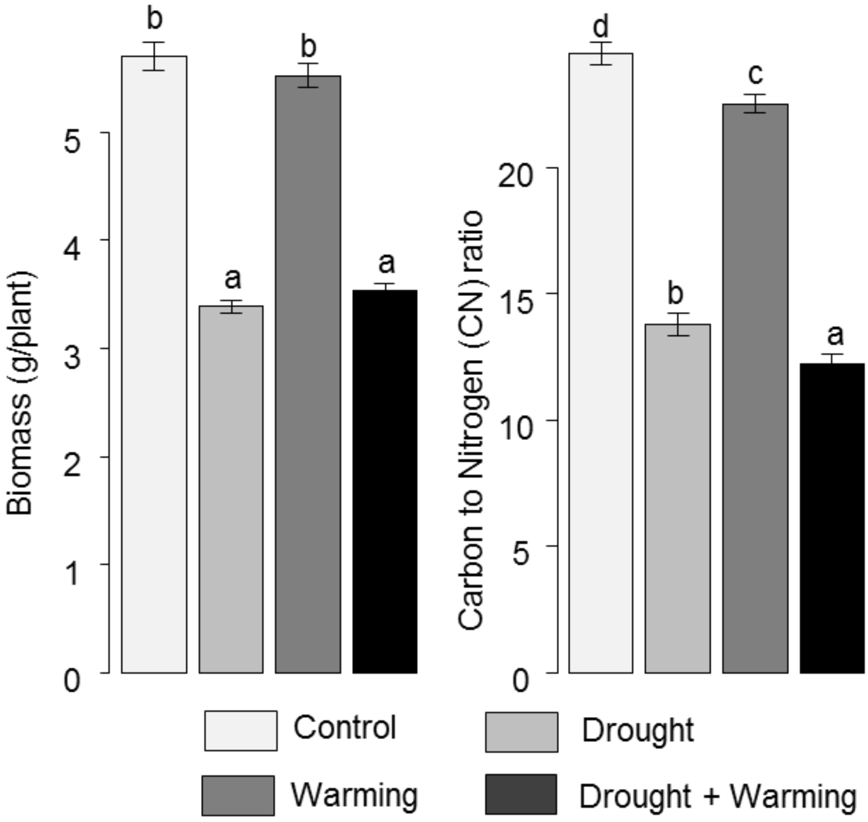
Plantago lanceolata

Trifolium pratense

Supporting information S4: Species and ecotype-specific treatment effects on each measured parameter and their interactions for drought and warming experiment (a) and for spring frost experiment (b). For the winter warming plus frost experiment (c) treatment effects on species and ecotypes of *A. elatius* as well as their interactions are presented. Linear mixed effects models were used to test treatment effects on all plant species and ecotypes with respect to the measured parameters (C/N ratio, biomass, chlorophyll content, percent greenness, green leaf biomass, dead tissue biomass, 15N uptake). Significant differences are marked in bold ($p < 0.05$).

Parameter	Response	F	P
(a) Drought and warming experiment			
Biomass (g)	Species	118.6	**<.0001**
	Ecotype	11.6	**<.0001**
	Drought	928.0	**<.0001**
	Warming	0.0	0.9001
	Species × Drought	8.4	**<.0001**
	Species × Warming	2.1	0.1043
	Drought × Warming	3.8	0.0527
	Ecotype × Drought	3.7	**0.0044**
	Ecotype × Warming	0.2	0.9474
	Species × Drought × Warming	0.9	0.4474
	Ecotype × Drought × Warming	0.5	0.7267
C/N ratio	Species	40.0	**<.0001**
	Ecotype	12.5	**<.0001**
	Drought	795.3	**<.0001**
	Warming	24.2	**<.0001**
	Species × Drought	2.3	0.0721
	Species × Warming	2.4	0.0704
	Drought × Warming	1.5	0.2256
	Ecotype × Drought	2.1	0.0596
	Ecotype × Warming	0.5	0.7838
	Species × Drought × Warming	2.5	0.0570
	Ecotype × Drought × Warming	0.7	0.6585
(b) Spring frost experiment			
Biomass (g)	Species	20.8	**<.0001**
	Ecotype	4.5	**0.0015**
	Spring frost	42.4	**<.0001**
	Species × Spring frost	3.5	**0.0160**
	Ecotype × Spring frost	0.6	0.6624
Chylorophyll content (spad)	Species	59.6	**<.0001**
	Ecotype	70.4	**<.0001**
	Spring frost	24.8	**<.0001**
	Species × Spring frost	9.8	**<.0001**
	Ecotype × Spring frost	8.6	**<.0001**
(c) Winter warming plus frost experiment			
Biomass (g)	Species	6.6	**<.0001**
	Ecotype	2.1	**0.0217**
	Treatment	54.2	**<.0001**
	Species × Treatment	1.2	0.3227
	Ecotype x Treatment	0.1	0.40833
Greenness (%)	Species	3.4	**0.0023**
	Ecotype	1.9	**0.0416**
	Treatment	67.9	**<.0001**
	Species × Treatment	1.5	0.1876
	Ecotype x Treatment	1.4	0.1123
Dead tissue biomass (g)	Species	8.4	**<.0001**
	Ecotypes	1.0	0.4422
	Treatment	35.1	**<.0001**
	Species × Treatment	2.3	**0.0091**
	Ecotype x Treatment	0.7	0.7887
Root ^{15}N uptake (mg label per g dry weight)	Species	14.3	**<.0001**
	Ecotypes	0.5	0.8177
	Treatment	6.2	**0.0027**
	Species × Treatment	2.2	**0.0236**
	Ecotype x Treatment	2.3	**0.0169**

Supporting information S5: Drought and warming experiment. Overall treatment effects on biomass and carbon to nitrogen ratio using pooled data from all species and ecotypes of the four grass species (see Table 2). Interactions between countries of origin / ecotypes, species and treatments are presented in supporting information S4a in Appendix.



Supporting information S6: Linear correlations between climate of origin and the mean relative responses of each ecotype for every species. Relative response was calculated as (control – treatment) / control. For the drought and warming experiment only the most significantly influential treatment was compared to control (drought). Climate parameters were acquired for each origin using variables downloaded from Worldclim (Hijmans *et al.*, 2005), using a resolution of 5 arc-seconds. Climate parameters (predictor variable) logically most probable to be correlated with the response parameters (explanatory variable) in each respective experiment were chosen. De Martonne’s aridity index: the ratio between the mean annual values of precipitation (P) and temperature (T) plus 10°C =P/(T+10) (De Martonne, 1926). Significant / close to significant correlations are marked in bold. De Martonne E., 1926. Une nouvelle fonction climatologique: L’indice d’aridité. La Meteorologie, 449-458.

Drought and warming experiment – response vs **de Marton aridity index**				
Parameter	Species	Slope	adjusted R^2	p
Biomass	*Arrhenatherum elatius*	-5,51 x 10^{-4}	-0,33	0,95
	Alopecurus pratensis	7,52 x 10^{-5}	-0,50	0,98
	Festuca pratensis	**0,007**	**0,42**	**0,14**
	Holcus lanatus	-1,7 x 10^{-4}	-0,50	0,97
C:N ratio	*Arrhenatherum elatius*	0,018	-0,08	0,46
	Alopecurus pratensis	0,006	0,50	0,18
	Festuca pratensis	0,008	0,34	0,18
	Holcus lanatus	**0,005**	**0,84**	**0,06**
Spring frost experiment - response vs minimum temperature in May				
Biomass	Biomass			
	Arrhenatherum elatius	-0,017	0,38	0,16
	Alopecurus pratensis	-0,009	-0,41	0,76
	Festuca pratensis	-0,008	-0,05	0,43
	Holcus lanatus	-0,018	-0,17	0,53
Chlorophyl content	*Arrhenatherum elatius*	0,010	-0,31	0,84
	Alopecurus pratensis	-0,004	-0,15	0,51
	Festuca pratensis	-0,009	-0,23	0,65
	Holcus lanatus	-0,015	0,22	0,31
Warming plus frost experiment – response vs minimum winter temperature				
Percent greenness	*Arrhenatherum elatius*	**-0,030**	**0,17**	**0,10**
Root N uptake	*Arrhenatherum elatius*	**-0,074**	**0,45**	**0,03**
Dead tissue biomass	*Arrhenatherum elatius*	0,290	-0,045	0,48
Biomass	*Arrhenatherum elatius*	**-0,044**	**0,35**	**0,02**

6.6 Manuscript 6

Title: Drought effects in climate change manipulation experiments influenced by ambient weather conditions – effect sizes and rain-out shelter artifacts

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Key words: Precipitation manipulation experiment, experimental design, artefact control, extreme weather event, EVENT-experiment, micro-climate, pulse experiment, drought

Abstract

Extreme drought events pose challenges to ecosystem functioning. Ecological response to drought is studied worldwide in a growing number of field experiments by the use of rain-out shelters. Yet the few emerging meta-analyses face severe challenges in the comparability of considered studies. This is in part because build-up of drought stress in rain-out shelter manipulations is modified by ambient weather conditions outside the shelters. Rain-out shelters are further known for creating confounding effects on radiation and temperature, which may influence plant responses. Yet, a detailed quantification of the development of drought effects within rain-out shelters under opposing outside weather conditions and of micro-climatological artifacts is missing.

Here, we examined the responses of phytometers - potted individuals of *Plantago lanceolata* of standardized size, age, pot size, substrate and initial soil moisture for each experimental run - under rain-out shelter, rain-out shelter artifact control, and ambient control during opposing outside micro-climatological conditions. Furthermore, we tested shelter artifacts on plant responses and whether the effect of drought manipulations by rain-out shelter on plant responses in a long term field experiment can be explained by ambient micro-climate.

Phytometers below the rain-out shelters showed significantly lower stomatal conductance, lower effective quantum yield, and lower leaf water potential during warm outside ambient weather conditions with high evaporative demand than during cold conditions with low evaporative demand. Plant performance was highly correlated with both single and multiple effects of ambient outside micro-climate conditions. Rain-out shelter artifacts on plant responses were non-significant.

We conclude that drought manipulations using the rain-out shelter technique are strongly affected by ambient weather conditions. This finding has implications for the comparability among studies and even between years within single studies. Relating drought responses to continually measured micro-climatological parameters such as air temperature and vapor pressure deficit can facilitate meaningful comparisons among the numerous existing and upcoming climate change experiments.

Introduction

Extreme climatic events are predicted to increase in frequency and magnitude (Min and others 2011; IPCC 2013) and are expected to have strong ecological implications (Jentsch and others 2007; Knapp and others 2008; Smith 2011; Reichstein and others 2013). Manipulation experiments in the field are powerful tools for the causal understanding of ecological processes in the face of climate change (Beier and others 2012; Smith and others 2014). Ecological response to climatic extremes such as drought events is therefore currently studied worldwide in a growing number of field experiments by the use of rain-out shelters (see Supporting information S1).

The quality of an experimental analysis, however, depends on whether the effective variables are really considered (Hurlbert 1984) and whether the experimental manipulations create hidden treatments (Huston 1997). Rain-out shelters are often criticized for unwanted side-effects on microclimatic conditions such as reduced wind speed, altered radiation, warming, etc. (Fay and others 2000; English and others 2005). Moreover, it can be expected that the manipulation strength of rain-out shelter experiments depends on outside weather conditions, as high air humidity during rainfall events should also affect the manipulated plots. Yet, detailed quantification of such micro-climatological artifacts is largely missing.

Up to now, there is one formal meta-analysis published on the results of precipitation manipulations (Wu and others 2011), and this analysis focused on reduced and increased total rainfall amounts without distinguishing between chronic partial rainfall reductions and pulsed full exclusion drought events or the timing and frequency of precipitation. Furthermore, it has recently been shown that soil moisture fluctuations under ambient reference conditions cannot successfully predict soil CO_2 efflux in precipitation manipulations across numerous experimental datasets (Vicca and others 2014). Lack of comparability in drought definitions and response parameters among studies are major limiting factors for generalizations across ecosystems (Vicca and others 2012). However, we argue that comparability is further hampered by missing information on outside weather conditions during drought manipulations. In general, effective differences between rain-out shelter manipulations and ambient control conditions can only be achieved during rainy periods. These conditions, however, are accompanied by high air humidity which can, for instance, cause opening of stomata (Lange and others 1971) also in the drought manipulation. Any drought manipulation by rain-out shelters therefore probably depends on the ambient outside weather conditions. Effect sizes, however, are yet to be experimentally tested.

Generally, drought manipulations are realized in field experiments either as pulsed full exclusion (complete precipitation withdrawal for defined, rather short periods of time) or partial exclusion (rainfall reduction typically by 30%, 50%, or 65% over longer times) manipulations. Up to now, the majority of published studies investigated full exclusion drought events (40 cases in S1) in comparison to partial exclusions (17 cases). Full exclusion drought manipulations are realized by fixed rain-out shelters (e.g. Fay and others 2000; 25 cases in S1) or by mobile shelters which automatically close only during rainfall events (e.g. Dugas and Upchurch 1984; 15 cases in S1). The latter might appear less sensitive for treatment artifacts, yet both techniques share the major challenge of trying to create drought when ambient conditions are humid. Partial exclusion is realized by partly covering the plots with translucent stripes that take away a defined percentage from any ambient rainfall event by a fixed setting (Yahdjian and Sala 2002) for rather long periods.

Fixed rain-out shelters in particular are criticized for unwanted side-effects such as increased air temperature, decreased solar radiation, wind, and vapor pressure deficit (Dugas and Upchurch 1984; Fay and others 2000; English and others 2005). In addition, rain-out shelters reduce turbulent exchange (i.e. turbulent heat flux, transpiration and carbon-dioxide exchange), potentially resulting in increased levels of carbon dioxide under the shelters due to plant dark respiration. Besides this discussion, however, a crucial lack of evidence is in the quantification of such artifacts on plant performance by the help of additional artifact controls (i.e. plots irrigated with ambient precipitation below fully equipped rain-out shelters).

Here, we used a standardized phytometer approach to track plant ecophysiological responses under three climate treatments, i.e. rain-out shelter manipulation, rain-out shelter artifact control, and ambient control in an existing climate manipulation experiment (EVENT II, Grant and others 2014). We compared the build-up of water stress in plants during standardized drought manipulation but opposing outside ambient weather conditions. Furthermore, we investigated community productivity under the three climatic treatments in a long term field experiment. We hypothesized that (1) drought stress in rain-out shelter experiments will build up faster during warm outside ambient weather conditions with high evaporative demand than during cold conditions with low evaporative demand. This implies that the realized drought effects are directly related to easily measurable meteorological parameters which will eventually allow for standardizations and comparability between sites and years. Furthermore, we expected that (2) rain-out shelter artifacts of fixed roofs on plant performance are considerable in their effect sizes.

Materials and methods

Experimental site

The present research was carried out in the Ecological Botanical Garden of the University of Bayreuth, Germany (49˚55′19″N, 11˚34′55″E, 365 m). The short-term phytometer experiment in 2012 was conducted in the existing long-term field experiment Event II (Grant and others 2014). For reference, long-term productivity data from EVENT II is included. The EVENT II site was covered by a semi-natural grassland consisting of about 13 species m^{-2} and dominated by tall grasses, particularly *Alopecurus pratensis* L. (meadow foxtail). The regional climate is temperate and moderately continental, with a mean annual temperature of 7.9 °C (1971–2000). The annual precipitation of 724 mm (1971– 2000) has a bimodal distribution with a major peak in June/July and a second peak in December/ January (Foken 2007). Following a spring drought in 2012, the precipitation during the measuring period (May to July) was comparable to the climatological mean (see Supporting information S2). Annual temperature and precipitation of 8.6 °C and 698 mm, respectively, represent a moderately humid study year 2012 according to a De Martonne aridity index of 37.5.

Rain-out shelter manipulations

The climate treatments consisted of annually recurrent full exclusion pulsed drought and controls (treatment levels: rain-out shelter, rain-out shelter artifact control, and ambient control). The ambient control plots remained without climate manipulation throughout the entire drought manipulation period and received ambient rainfall. Identical rain-out shelters were set up on rain-out shelter artifact controls and rain-out shelter plots during the climate manipulation. Rain-out shelter plots remained without precipitation but rain-out shelter artifact plots received the same amount of precipitation as ambient control plots, added by irrigation from above the canopy after every rainfall. The shelters were constructed from a steel frame (Hochtunnel, E & R Stolte GmbH, Germany), and covered with a transparent plastic sheet (material: 0.2 mm polyethylene, SPR 5, Hermann Meyer KG, Germany, see Figure 1). Rain-out shelters permitted nearly 90% penetration of Photosynthetically Active Radiation. Greenhouse effects due to rain-out shelters were minimized by having an 80 cm clearance between the roof edge and the ground, allowing for near-surface air exchange. Identical or very similar shelters have been used by us for several studies (Jentsch and

Beierkuhnlein 2010) and also by other groups (e.g. Kahmen and others 2005; Fay and others 2000). Lateral surface flow was avoided by barriers of plastic sheet pilings around all plots reaching down to a depth of 20-25 cm.

Figure 1: The EVENT II experiment with the different precipitation manipulations (photo: C. Schaller).

Phytometer experiment

We used *Plantago lanceolata* as phytometer species. It is native to large parts of Eurasia, naturally common at the experimental site, and occurs as non-native weed in e.g. North America, South America and Australia (Seipel and others 2012). Moreover, this species has been used as model plant for stomatal conductance measurements by (Clark and others 1999) and as phytometer by Temperton et al. (2007). *P. lanceolata* was grown as temporal cohorts, so that they could be transferred to the field site at different dates, whilst being of identical age. Individuals of *P. lanceolata* were grown from seeds, using a standardized soil substrate: 20% washed sand, 20% fine lava (sand and lava- steamed at 90 °C), 60% white peat and black peat. The plants were germinated and grown in climate chambers under light (250 µmol m^{-2} s^{-1} PAR) at 20 °C for 15 hours and without light at 10 °C for 9 hours. All pots were watered on daily basis. After germination of seeds (20th day) we transplanted 30 single vigorous individuals into pots (9 x 9 x 9.5 cm^3), using sandy loam as soil substrate (82% sand, 13% silt, 5% clay; pH = 4.5 and total N = 0.07%). Pot size and soil substrate were selected according to pre-trials which showed that this volume and substrate would induce severe drought stress after seven days during warm and dry conditions. The first cohort of plants was sown on March 22nd, then, over the next five weeks another five sets of plants were grown for the 2nd, 3rd, 4th, 5th and 6th cohorts, each of 30 individuals. This sequential sowing ensured that each set of plants exposed to the experimental site was of the same age (62 days).

Before exposure in the field we soaked each pot in water for 2 min, to ensure the moisture balance was equal between pots. The first cohort of phytometers was taken out to the experimental site on 23rd of May, 2012 (see Supporting information S3 for detailed schedule).

In summary, contrasting outside ambient weather conditions during the experimental periods (23 May – 03 July, 2012) were evaluated by six temporal cohorts of phytometers, which were set under three climate manipulations (rain-out shelter, rain-out shelter artifact control, and ambient control). Each set of phytometers was exposed to the weather conditions in its respective climate treatments for seven days. We used 10 phytometers for each treatment per temporal cohort in a nested block design (pots located in center of shelter with app. 20 cm distance to each other) and 30 replicates for each temporal cohort.

Response parameters in the phytometer experiment

Stomatal conductance, effective quantum yield of photosystem II (ΔF/Fm'), and leaf water potential are frequently studied response parameters for tracking drought stress in plants. Stomatal conductance and ΔF/Fm' can respond quickly within hours, whereas leaf water potential reacts more slowly, typically within days.

Stomatal conductance (mmol m^{-2} s^{-1}) was measured during midday (12:00 - 2:00 pm, maximum stress) at the center of a medium aged leaf from each plant using a steady state leaf porometer (SC-1, Decagon Devices, Inc) at 1st day, 2nd day, 3rd day, and 7th day of exposure per cohort in the rain-out shelters (see Supporting information S3 for actual dates).

Dark adapted chlorophyll fluorescence was measured at mid night (12:00 – 1:00 am) once per cohort (7th day) at the center of medium aged leaves using a PAM 2000 portable chlorophyll Fluorometer (Heinz Walz, Germany). The effective quantum yield of photosystem II was quantified as ΔF/Fm'= (Fm'-Ft)/Fm' where Ft and Fm' are the actual and maximal chlorophyll fluorescence under ambient conditions, respectively (Genty and others 1989). ΔF/Fm' reflects the efficiency of light energy conversion of photosystem II (PS II) which is a sensitive parameter to quantify stress effect and photoinhibition in plants (Bolhar-Nordenkampf and others 1989; Werner and others 2002). Leaves were measured in situ in the natural position with a leaf clip holder after Ft had stabilized (about 20 seconds). To enable a comparison among absolute fluorescence values, a fluorescence standard foil was measured before each measuring cycle. Standard measurements were used to normalize the fluorescence values obtained and to calibrate the PAM in use.

Leaf water potential (MPa) was measured on the 7th day (maximum stress) with a PMS 600 pressure bomb (PMS Instrument Company, Albany, USA) using the Scholander pressure bomb technique. One medium aged leaf from each plant was placed inside the closed chamber and pressurized.

Response parameters in the long term field experiments

We investigated annual net primary production (ANPP g m^{-2}) and total cover (%) in ambient control versus rain-out shelter artifact control in the main field experiment (EVENT II, see Grant et al. 2014 for details) over four consecutive years (2009-2012) to quantify long-term shelter artifacts.

Ambient microclimate parameters used to explain the drought effects in plant performance

In order to investigate the relationship between ambient weather conditions outside the rain-out shelters and the degree of drought stress for the six temporal cohorts in the phytometer experiment, we investigated three easily measurable micro-climate parameters, i.e. air temperature (°C), vapor pressure deficit (*VPD*, hPa), and incoming shortwave radiation (R_{sw}, Wm^{-2}). Air temperature and humidity were measured by a Frankenberger Psychrometer (Friedrichs & Co., Germany), radiation measurements (upwelling and downwelling longwave and shortwave components) with a CNR1 net radiometer (Kipp & Zonen, Netherlands). VPD was calculated from the difference between saturation water vapour pressure (hPa) and actual water vapour pressure (hPa). More details about these measurements are given by Babel et al. (2013). In addition, soil moisture in the main rooting zone (between 5 and 10 cm depth) was recorded by FD-sensors (Echo.EC-5 ⁄ k; Decagon Devices, Pullman,WA, USA) in the long term EVENT II experiment.

Data analysis

Two factorial linear mixed-effects models combined with analysis of variance (ANOVA) were applied to test for significant differences between cohorts and treatments for all parameters in the phytometer experiment. The two fixed factors climate manipulation (rain-out shelter, rain-out shelter artifact control, and ambient control) and outside weather conditions (six cohorts) were tested for their single and interactive effects. The blocked spatial configuration of the experimental design was acknowledged in the mixed models by inserting the row and column of the treatment blocks as random effects. Prior to statistical analysis, data were power or log transformed to improve the homogeneity of variances, or if conditions of normality were not met. Both characteristics were tested by examining the residuals versus fitted plots and the normal qq-plots of the linear models. Homogeneous groups of factor combinations were identified by Tukey HSD post hoc comparisons. Level of significance was set to $p < 0.05$.

Correlation analyses between ambient microclimate parameters outside rain-out shelters and plant responses inside rain-out shelters were conducted in order to link the drought effects to various ambient weather conditions. We used three microclimate parameters (air temperature, vapor pressure deficit, and incoming shortwave radiation) and three plant responses (stomatal conductance, ΔF/Fm’ of photosystem II, and leaf water potential) for correlation analysis as these parameters were significantly different among the six cohorts, or at least there was a trend (incoming shortwave radiation) (Table 1).

Furthermore, we tested for long-term rain-out shelter artifacts on ANPP and cover responses by comparing ambient control and rain-out shelter artifact control over four consecutive years (2009-2012) in a semi-natural temperate grassland (EVENT II, Grant et al. 2014). A linear mixed effect model combined with ANOVA was used to test for significant differences between years and treatments. Homogeneous groups of factor combinations were identified by Tukey HSD post hoc comparisons.

All analyses were run in R version 3.1.2 (R Core Team 2014) with the additional packages multcomp version 1.3-2 (function glht), nlme version 1.0-6 (function lme), and sciplot version 1.1-0 (function bargraph.CI).

Table 1: Mean daily ambient micro-climate parameters outside rain-out shelters within the six cohorts. Cohorts and years are ordered from low to high temperature. Significant differences among cohorts ($p < 0.05$) and years are marked in bold. “Air temp” = Air temperature; “VPD” = Vapor pressure deficit; “R_{sw}” = Incoming shortwave radiation.

Phytometer experiment	**Microclimate**	**Cohort 2**	**Cohort 3**	**Cohort 5**	**Cohort 1**	**Cohort 4**	**Cohort 6**	**F**	**p**
	Air temp. (°C)	14.8	16.6	18.5	19.4	19.7	21.2	**3.5**	**0.013**
	VPD (hPa)	5.9	7.0	8.9	12.3	9.3	10.6	**2.5**	**0.049**
	R_{sw} (Wm^{-2})	283	333	388	436	377	370	2.0	0.104

Results

Ambient weather conditions outside rain-out shelters drive drought effect

The phytometers inside the rain-out shelters exhibited significantly reduced stomatal conductance ($F = 42.2$; $p < 0.001$), effective quantum yield ($F = 43.8$; $p < 0.001$) and leaf water potential ($F = 26.3$; $p < 0.001$) during warm outside weather conditions with high evaporative demand as compared to during cool conditions with low evaporative demand (Fig.2). Drought compared to ambient control significantly reduced plant stomatal conductance, ΔF/Fm’ of photosystem II, and leaf water potential only when outside ambient weather condition were warm and dry (Cohorts 4, 5, 6; Fig. 3). The rain-out shelter manipulation did not significantly hamper the three plant response parameters compared to control in cool cohorts with low evaporative demand (in particular Cohort 2, same trend in Cohort 3; Fig. 3). The clear pattern of drought effects under the rain-out shelters developing only under warm outside weather conditions with high evaporative demand (Fig. 2), however, is also confounded by the fact that significant treatment effects (the comparison between rain-out shelter manipulation and ambient control) further depend on the amount of precipitation taken away by the shelters. The pattern of drought treatment effects in Figure 3 therefore also depends on sufficient precipitation in the ambient controls to create any difference in moisture availability among the treatments. High temperatures combined with high evaporative demand cannot result in a significant difference among the rain-out shelter manipulation and the ambient control if there is no effective difference in precipitation, as clearly shown by cohort 1 (Fig. 3).

The drought effects as observed in the ecophysiological plant responses were strongly correlated with the ambient microclimate if considered over several days (Table 2): Stomatal conductance was significantly correlated with the microclimate during phytometer exposure from the second day onwards, while it was not significantly related to any microclimatic parameter after exposure for only one day (Table 2). Likewise, effective quantum yield of photosystem II (ΔF/Fm') was significantly correlated with outside ambient microclimate conditions. Again, this relationship was stronger if considered over longer time spans (climate during the full cohort exposure versus climate of the final day of exposure only, Table 2). ΔF/Fm' and also leaf water potential were not significantly correlated with incoming shortwave radiation over the full cohort exposure. However, water potential was strongly related to the microclimate during the full exposure time. In general, multiple regressions, in particular those using temperature and VPD resulted in the strongest correlations across all response parameters.

Rain-out shelter artifacts

Microclimatic parameters were considerably altered by the rain-out shelters with mean midday shortwave radiation being reduced by 22% and net radiation by 18% (Table 3). Mean summer temperatures were less strongly influenced. Still, mean day time air temperature was reduced by 1.1°C while night time temperature increased by 0.4°C (soil temperatures going into the same direction, Table 4). Soil moisture showed no consistent rainout shelter artifact (slight increases or decreases in mean manipulation time soil moisture among years between artifact control and ambient control, Table 4). Minimum temperatures, however, were considerably higher under the shelters than in ambient control conditions (except mid-day, see Table 5).

Stomatal conductance, ΔF/Fm', and leaf water potential in the rain-out shelter artifact treatment did not significantly differ from the ambient control (Fig. 4). Furthermore, ANPP and aboveground plant cover of ambient control vs. rain-out shelter artifact control did not significantly differ and no significant interaction between treatment and year was observed in the long term field experiment (EVENT II, Fig. 5).

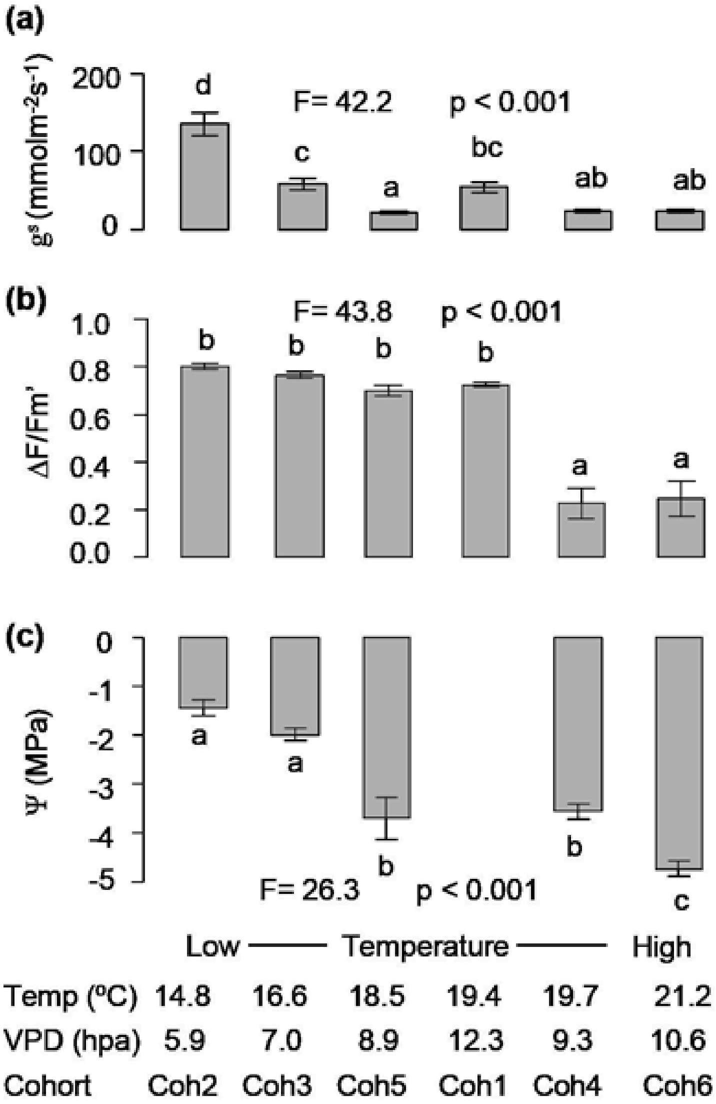
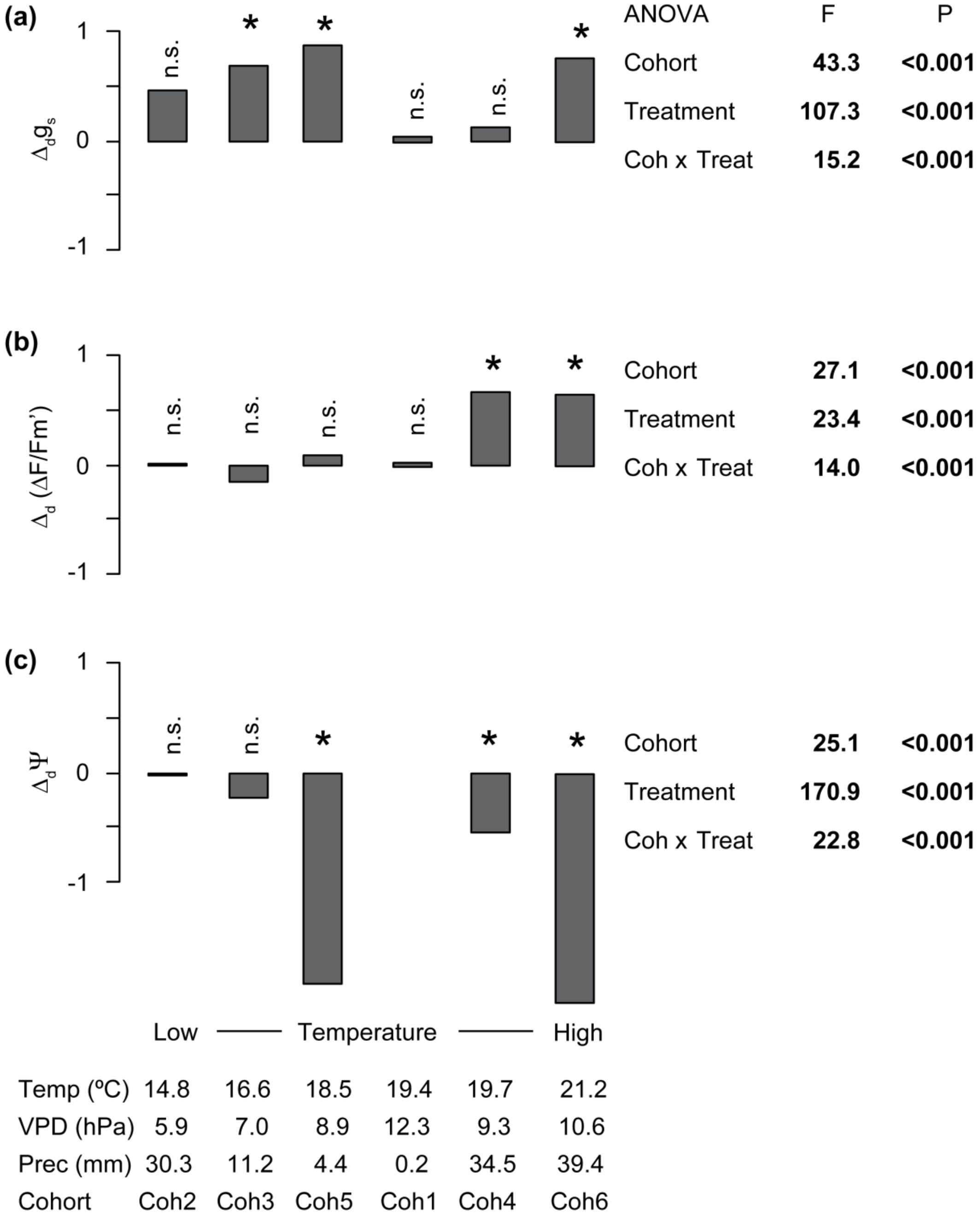


Figure 2: Ecophysiological responses of standardized phytometer plants to exposure under rain-out shelters for seven days under different outside weather conditions (as summarized below the graph, daily mean of 30 min values during daytime). (a) stomatal conductance (g_s), (b) effective quantum yield of photosystem II (ΔF/Fm’), (c) leaf water potential (Ψ). 7th day mean and standard error of 10 replicates per cohort are shown. Same letters indicate homogeneous groups according to TukeyHSD post-hoc comparisons.



ANOVA	F	P
Cohort	**43.3**	**<0.001**
Treatment	**107.3**	**<0.001**
Coh x Treat	**15.2**	**<0.001**

Cohort	**27.1**	**<0.001**
Treatment	**23.4**	**<0.001**
Coh x Treat	**14.0**	**<0.001**

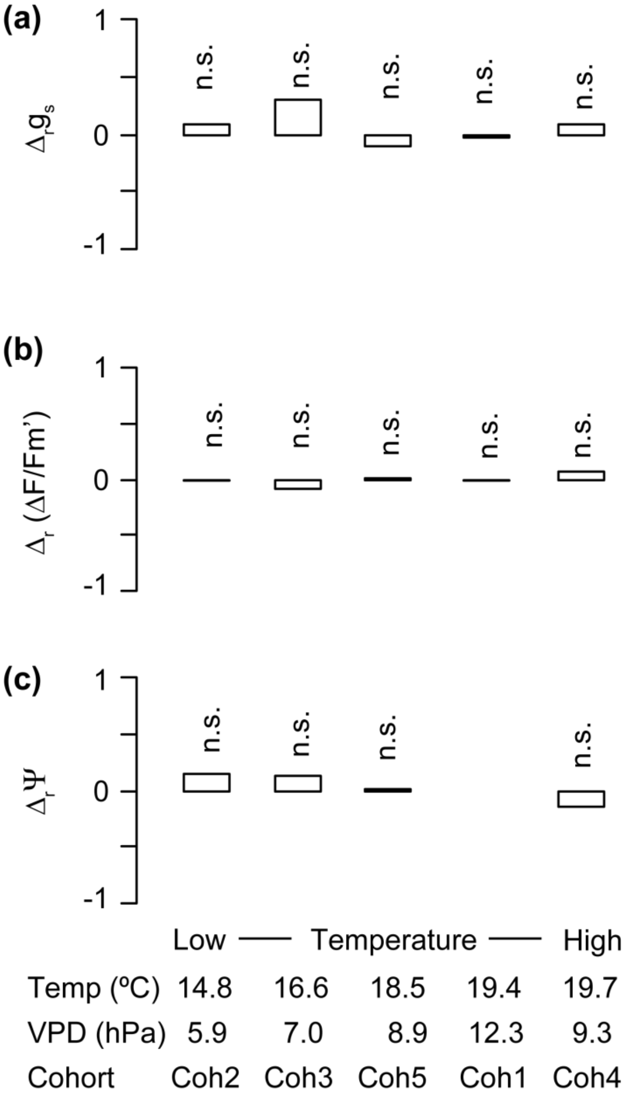
Cohort	**25.1**	**<0.001**
Treatment	**170.9**	**<0.001**
Coh x Treat	**22.8**	**<0.001**

Temp (ºC)	14.8	16.6	18.5	19.4	19.7	21.2
VPD (hPa)	5.9	7.0	8.9	12.3	9.3	10.6
Prec (mm)	30.3	11.2	4.4	0.2	34.5	39.4
Cohort	Coh2	Coh3	Coh5	Coh1	Coh4	Coh6

Figure 3: The relative differences in performance of standardized phytometer plants between ambient control and the rain-out shelter manipulation (Δ_d) after seven days of exposure for (a) stomatal conductance (g_s), (b) effective quantum yield of photosystem II (ΔF/Fm'), and (c) leaf water potential (Ψ) for six temporal cohorts differing in outside weather conditions (as summarized below the graph, daily mean of 30 min values during daytime). Significant results ($p < 0.05$) of the corresponding two-factorial ANOVA with cohort, treatment, and their interaction as fixed effects are marked in bold and significant pair wise comparisons between ambient control and drought in each cohort according to TukeyHSD post hoc comparisons are indicated by asterisks. "Coh" = Cohort; "Treat" = Treatment. Daily means of air temperature ("Temp"), vapor pressure deficit ("VPD"), and sums of precipitation ("Prec") are given per cohort.

Table 2: Relationship between phytometer drought stress (plant physiological responses) and ambient micro-climate conditions outside rain-out shelters in an ecosystem drought manipulation experiment. Note that multiple regression against all three microclimatic parameters were not assessed due to limited sample size (n = 6). Given are adjusted r²-values of ordinary least squares regressions if $p < 0.05$.

	Temp mean		**VPD mean**		**R_{sw} mean**		**Multiple regression (Temp × VPD)**		**Multiple regression (Temp × R_{sw})**		**Multiple regression (VPD × R_{sw})**		**Multiple regression (Temp + VPD +Rsw)**	
	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p
Stomatal conductance Vs Micro-climate														
1st day mean vs 1st day 3 hours average		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.
1st day mean vs 1st day average		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.
2nd day mean vs 2nd day 3 hours average		n.s.		n.s.	0.63	0.036		n.s.	0.93	0.039		n.s.		n.s.
2nd day mean vs 2nd day average		n.s.		n.s.	0.65	0.033		n.s.	0.98	0.010	0.98	0.011		n.s.
2nd day mean vs first 2 days average		n.s.		n.s.		n.s.	1.00	0.006	1.00	0.001	0.98	0.014		n.s.
3rd day mean vs 3rd day 3 hours average		n.s.	0.76	0.015	0.82	0.008	0.99	0.008	0.69	0.026	0.97	0.016	0.81	0.009
3rd day mean vs 3rd day average		n.s.	0.64	0.034	0.65	0.033	0.94	0.037	0.92	0.050		n.s.	0.63	0.036
3rd day mean vs first 3 days average		n.s.	0.80	0.010	0.92	0.001	0.94	0.033	0.96	0.022	0.99	0.008	0.94	0.001
7th day mean vs 7th day 3 hours average	0.74	0.018		n.s.		n.s.	0.99	0.009	0.99	0.007		n.s.		n.s.
7th day mean vs 7th day average	0.75	0.017		n.s.		n.s.	0.96	0.023	1.00	0.001		n.s.		n.s.
7th day mean vs full 7 days average	0.70	0.023		n.s.	0.58	0.049	0.96	0.024	0.99	0.004	0.99	0.003	0.97	0.020
Effective quantum yield Vs Micro-climate														
7th day mean vs 7th day average	0.58	0.048		n.s.		n.s.	0.97	0.017		n.s.		n.s.	0.96	0.021
7th day mean vs full 7 days average	0.81	0.010	0.58	0.049		n.s.	1.0	0.002	0.93	0.039	0.95	0.028	0.99	0.004
Water potential Vs Micro-climate														
7th day mean vs first 4 days average	0.86	0.015	0.75	0.037	0.90	0.010	0.81	0.025	0.90	0.009	0.85	0.016	0.92	0.007
7th day mean vs first 5 days average	0.89	0.010	0.84	0.018	0.91	0.008	0.86	0.015	0.92	0.006	0.90	0.009	0.91	0.008
7th day mean vs first 6 days average	0.99	0.001	1.0	<.001	0.91	0.008	1.0	<.001	0.94	0.004	0.97	0.001	0.85	0.016
7th day mean vs full 7 days average	0.93	0.005	0.98	0.001		n.s.	0.95	0.003	0.86	0.015	0.90	0.009	0.99	0.050



ANOVA	F	P
Cohort	**57.6**	**<0.001**
Treatment	1.4	0.234
Coh x Treat	0.4	0.837

Cohort	**17.7**	**<0.001**
Treatment	0.5	0.503
Coh x Treat	1.6	0.191

Cohort	**60.8**	**<0.001**
Treatment	0.0	0.888
Coh x Treat	2.7	0.057

Figure 4: The relative differences in performance of standardized phytometer plants between ambient control and rain-out shelter artifact control (Δ_r) after seven days of exposure for (a) stomatal conductance (g_s), (b) effective quantum yield of photosystem II (ΔF/Fm'), and (c) leaf water potential (Ψ) during five temporal cohorts differing in outside weather conditions (as summarized below the graph, daily mean of each 30 min values during daytime). Note that the sixth cohort is not displayed due to failed irrigation in the rain-out shelter artifact control. Significant results ($p < 0.05$) of the corresponding two-factorial ANOVA with cohort, treatment, and their interaction as fixed effects are marked in bold. Note that ambient control and rain-out shelter artifact control did not significantly differ in any response parameter (Treatment in ANOVA tables) and that furthermore no significant interaction between treatment and cohort was observed. "Coh" = Cohort; "Treat" = Treatment; "Temp" = Air temperature; "VPD" = Vapor pressure deficit.

Table 3: The relative differences in mean midday radiation (Wm^{-2}) between ambient control and rain-out shelter artifact control in phytometer experiment. Mid-day mean of each 30 min values during 10 am to 2 pm (CEST, without sunlight saving time) are shown here. Radiation was measured by a fully automatic horizontal mobile measuring system (HMMS, see Babel et al. 2013 for details). Relative difference (%) = (control – drought)*100/control. Positive differences (%) indicate radiation is higher in ambient control.

Microclimate	**Cohort 2**	**Cohort 3**	**Cohort 5**	**Cohort 1**	**Cohort 4**	**Cohort 6**
Mean midday incoming shortwave radiation (Wm^{-2})						
Ambient control	440	689	611	741	576	765
Shelter artifact control	336	533	487	597	455	591
Relative difference	24%	23%	20%	19%	21%	23%
Standard deviation for ambient control	±135	±187	±174	±157	±179	±115
Mean midday net radiation (Wm^{-2})						
Ambient control	298	469	423	495	396	534
Shelter artifact control	236	382	352	427	330	434
Relative difference	21%	19%	17%	14%	17%	19%
Standard deviation for ambient control	±102	±135	±135	±121	±134	±89

Table 4: Long-term differences in mean temperature and soil moisture between ambient control and rain-out shelter artifact control in EVENT II experiment. Mean day (6:00 to 22:00 CEST) and night (23:00 to 5:00, CEST) temperature of each 60 min values during the experimental climate manipulation (42 days during Mid May – June each year) are shown here. Temperature was measured at +5cm (with radiation shield) and at -2cm (top soil). Positive values indicate ambient control is higher and negative values indicate rain-out shelter artifact control is higher.

EVENT II	Summer 2009	Summer 2010	Summer 2011	Summer 2012
Mean day time temperature (°C) at +5 cm				
Ambient control	-	17.7	18.0	19.2
Shelter artifact control	-	16.2	17.2	18.2
Difference	-	+1.5	+0.8	+1.0
Mean night time temperature (°C) at +5 cm				
Ambient control	-	12.1	12.4	12.5
Shelter artifact control	-	12.3	12.9	13.0
Difference	-	-0.2	-0.5	-0.5
Mean day time temperature (°C) at -2 cm				
Ambient control	-	16.9	17.6	18.2
Shelter artifact control	-	15.7	16.6	17.6
Difference	-	+1.2	+1.0	+0.6
Mean night time temperature (°C) at -2 cm				
Ambient control	-	13.5	13.3	14.0
Shelter artifact control	-	13.5	14.1	14.0
Difference	-	0	-0.8	0
Soil moisture % (-5 to -10 cm)				
Ambient control	29.2	32.2	25.3	13.8
Shelter artifact control	27.2	39.7	24.0	09.1
Difference	+2.0	-7.5	+1.3	+4.7

Table 5: Differences in minimum temperature between ambient control and rain-out shelter artifact control in EVENT II experiment. Minimum morning (5:00 to 9:00 CEST), mid-day (10:00 to 14:00 CEST), evening (15:00 to 22:00 CEST) and mid-night (23:00 to 4:00 CEST) temperature of each 60 min values during the experimental climate manipulation (42 days during Mid May – June each year) are shown here. Temperature was measured at +5cm (with radiation shield) and at -2cm (top soil). Positive values indicate ambient control is higher and negative values indicate rain-out shelter artifact control is higher.

EVENT II	Summer 2010	Summer 2011	Summer 2012
Minimum morning temperature (°C) at +5 cm			
Ambient control	6.6	5.9	6.2
Shelter artifact control	8.1	6.6	8.3
Difference	-1.5	-0.7	-2.1
Minimum mid-day temperature (°C) at +5 cm			
Ambient control	8.6	12.1	12.3
Shelter artifact control	8.5	10.9	12.0
Difference	+0.1	+1.2	+0.3
Minimum evening temperature (°C) at +5 cm			
Ambient control	8.9	8.6	8.4
Shelter artifact control	8.9	10.0	8.9
Difference	0	-1.4	-0.5
Minimum mid-night temperature (°C) at +5 cm			
Ambient control	6.9	6.1	6.2
Shelter artifact control	7.8	6.8	7.2
Difference	-0.9	-0.7	-1.0
Minimum morning temperature (°C) at -2 cm			
Ambient control	8.9	7.6	9.4
Shelter artifact control	9.0	9.0	10.1
Difference	-0.1	-1.4	-0.7
Minimum mid-day temperature (°C) at -2 cm			
Ambient control	9.5	12.7	12.2
Shelter artifact control	9.3	11.5	12.2
Difference	+0.2	+1.2	0
Minimum evening temperature (°C) at -2 cm			
Ambient control	9.7	10.3	11.4
Shelter artifact control	9.5	11.5	11.0
Difference	+0.2	-1.2	+0.4
Minimum mid-night temperature (°C) at -2 cm			
Ambient control	8.9	7.7	9.0
Shelter artifact control	9.0	9.2	9.3
Difference	-0.1	-1.5	-0.3

Discussion

Ambient weather conditions outside rain-out shelters drive drought manipulation effects

Drought stress inside rain-out shelters built up only during dry outside conditions with high evaporative demand, not during cold outside conditions with low evaporative demand in our experiment. This dependence of drought manipulation effects on ambient weather conditions implies that insights from different experimental sites and even from different years within the same experiment are hardly comparable, as they depend on uncontrollable weather patterns. Generalizations of the effects of full exclusion drought events just based on drought length of rain-out shelter studies are therefore elusive. Field scale precipitation manipulation experiments remain, however, a crucial tool for our causal understanding of ecosystem responses to climate change (Beier and others 2012) and are applied in increasing numbers (Supporting information S1).

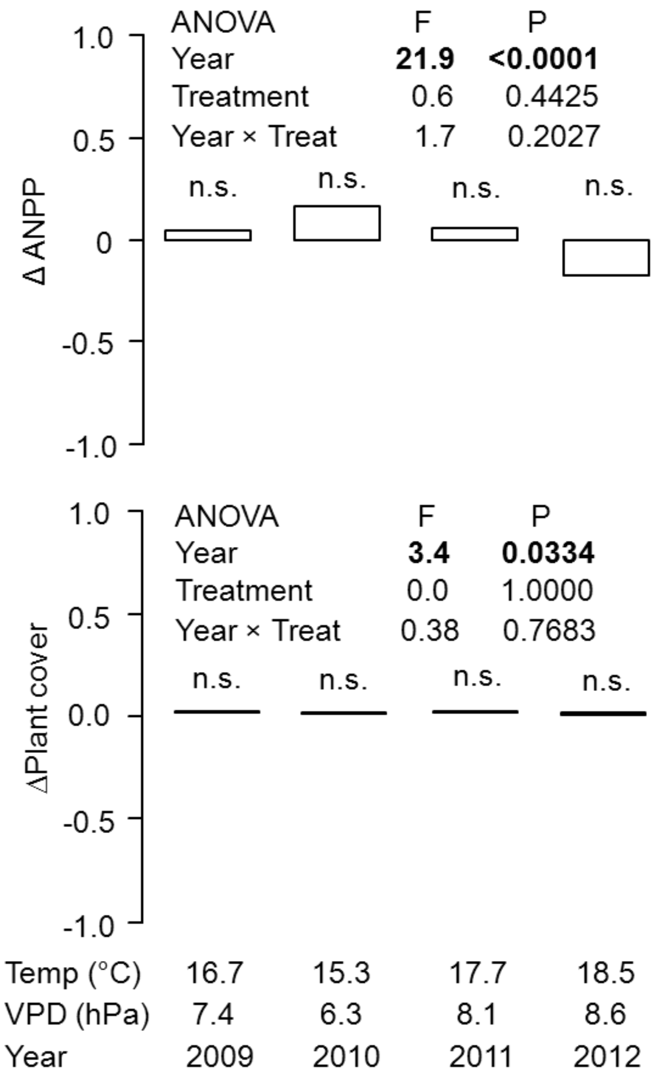


Figure 5: The relative differences in plant responses between ambient control and rain-out shelter artifact control over four years (2009-2012) in a semi-natural temperate grassland (EVENT II, n = 5 except for ANPP responses in 2012 where n = 4). Significant results ($p < 0.05$) are marked in bold. Ambient daytime mean temperature (Temp), and vapor pressure deficit (VDP) over the experimental run (42 days during May to July each year) are given here.

Temperature and VPD scales drought effects on plant performance

The strong correlation between the realized ecophysiological drought effects and the ambient microclimate may offer options for generalizations: At least for our experiment, drought effects can be standardized by microclimate during the drought manipulations and are then comparable among years. This relation needs to be tested under various climatic settings in other locations in order to test if it may offer a general option for facilitating comparability among drought manipulations. Up to now, many studies report on the air temperature during drought manipulations, while VPD is rarely mentioned in any paper (Supporting information S1). VPD, which basically measures the dryness of the air, has been shown repeatedly to be closely correlated with plant productivity in systems ranging from tropical rainforests (Brando and others 2010) to maize monocultures (Kiniry and others 1998) or the understory of temperate forests (Leuschner 2002). Meta-analyses will benefit from the reporting of outside conditions such as temperature and VPD as they need to explore responses to more complex and more realistic scenarios (Kreyling and Beier 2013) than simple annual reduction of rainfall (Wu and others 2011).

The effect of outside weather conditions on plant performance inside rain-out shelters in full exclusion drought manipulations depends, of course, on water availability and therefore on soil water status. We favor the quantification of easily measurable microclimatic parameters such as air temperature and VPD, in combination with rainfall quantification, for comparisons among and within studies because the soil water status depends on a multitude of parameters (Hickler and others 2009) which can be quantified only with considerable effort. Soil water status depends strongly on soil type, soil structure, soil depth, plant identity and plant performance, just to name a few of the interacting variables. All of these parameters are spatially highly heterogeneous and temporally highly dynamic (Bogner and others 2008; Glaser and others 2013). In particular in light of the current effort for coordinated distributed experiments with low maintenance effort and high spatial replication (Knapp and others 2012; Fraser and others 2013), such detailed quantification of soil water status is hardly feasible. We therefore recommend the quantification of ambient microclimate, which might even be taken *a posteriori* from nearby weather stations, in order to compare and generalize from a multitude of studies.

Here, we only considered a full exclusion pulsed drought manipulation experiment, i.e. the complete withdrawal of precipitation over rather short periods of time (e.g. Jentsch and others 2007; Jentsch and others 2011). Yet also the magnitude of manipulation effects in partial rainfall exclusions with rain-out shelters equipped with strips covering and discharging from only parts of the surface covered by the shelter (Yahdjian and Sala 2002) depends strongly on outside conditions: during wet years, their effect may equal an average year, while during a dry year they create extreme conditions. Meaningful manipulation periods therefore require decades rather than single years in partial rainfall exclusion experiments (Kreyling and Beier 2013). Taken together, full and partial rainfall exclusions are not experimental alternatives, but rather mutual aspects of a larger picture. In order to allow for comparability of the effectiveness of full and partial rainfall exclusions, we recommend the quantification of ambient microclimate with meaningful temporal (e.g. daily) resolution in either approach.

Rain-out shelter artifacts on plant responses

We found no significant rain-out shelter artifacts neither in short-term ecophysiological nor in long-term productivity and cover responses compared to ambient conditions (Fig. 4 and 5) despite considerable direct effects on micro-climate (Tables 3, 4, 5). For short-term full exclusion manipulations, this finding implies that fixed shelters can be used without creating strong artifacts with respect to the response parameters used in this study. Furthermore, the obvious micro-climatological effects are in the direction of natural drought events, which are commonly accompanied by increased air temperatures (De Boeck and others 2010). It needs to be kept in mind, though, that the elimination of early-morning late frost events by the capture of long-wave outgoing radiation might create a hidden treatment *sensu* Huston (1997). Such frost events can eventually cause drastic declines in grassland productivity (Kreyling and others 2012).

Movable shelters which automatically close only during rainfall events are a technically advanced solution which avoids artifacts outside rainfall events (Beier and others 2004). Investment costs and their need for electricity, however, oppose their widespread use. Furthermore, these shelters usually close quite slowly and often after the onset of rainfall events and are not always functional during stormy conditions (Beier et al. 2004). A complete withdrawal of water input is therefore hardly achieved, particularly in the face of short and intense rainfall events such as thunderstorms which typically carry most of the water input during growing seasons in temperate climates.

Partially covered rain-out shelters as commonly used for the manipulation of chronic rainfall reductions can be expected to cause similar, yet weaker micro-climatic artifacts (Yahdjian and Sala 2002). The typical duration of manipulations for several years, however, raise concerns that even small micro-climatic artifacts such as shading and warming might affect the ecological processes in the long run. Applying the same shelters with stripes turned upside down and therefore discharging into the shelters for control plots can circumvent these artifacts and allow for sound comparisons within studies. However, caution is required when the results are up-scaled to real-world conditions where dry periods are rather accompanied by increased incoming radiation than by shading (e.g. Brando and others 2010).

Conclusions

We conclude that the effectiveness of drought simulations by rain-out shelters depends strongly on ambient outside weather conditions. Drought stress is only realized during warm and dry outside conditions. This finding limits the comparability of rain-out shelter studies in space and time. The close relation between drought stress and easily measurable outside microclimatic variables such as air temperature and vapor pressure deficit at our study site, however, might allow for standardization among studies and years, and therefore for comparability and generalizations. Yet it needs to be tested at other locations. Fixed rain-out shelters used for short-term manipulations of full exclusion drought events create less impact on the plant responses than previously thought. Fixed shelters therefore remain a valid alternative to expensive automatically moved shelters. Rain-out shelters are and will remain a crucial tool for ecological climate impact research. Quantification of outside micro-climate and knowledge about micro-climatic artifacts and hidden treatments will secure comparability among studies and sound up-scaling and generalizations.

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Supporting information S1: Drought manipulation experiments based on an ISI Web of Science search in March 2014 with the search string (drought OR climate* extreme) AND "field experiment*" and the additional conditions that the drought was created by rain-out shelters and that multi-species systems were studied (as opposed to agricultural monocultures) in the field (not in pots). The search was expanded by expert knowledge. Time frame: 2000 -2014.

Rain-out shelter	**Drought type**	**Drought effects on response parameters**	**Microclimatic conditions mentioned to have been measured (often not reported as data)**	**Vegetation type**	**Source**
Fixed	full exclusion & partial exclusion	Increased temporal variation in rainfall: Aboveground net primary productivity ↓, soil CO_2 flux ↓, flowering duration ↓. Partial exclusion reduction: no effect	Air temperature, VPD, PAR, Net radiation,	Grassland	Fay et al. (2000)
Fixed	full exclusion & partial exclusion	Increased temporal variation in rainfall affects ecophysiology of plants as strong as reduced total rainfall amount	Air temperature, VPD, Net radiation,	Grassland	Fay et al. (2002)
Fixed	full exclusion & partial exclusion	Increased temporal variation in rainfall decreases carbon flux stronger than reduced total rainfall amount	NA	Grassland	Harper et al. (2005)
Fixed	full exclusion	Above-ground productivity ↓; Below-ground productivity ↑	Air temperature	Grassland	Kahmen et al. (2005)
Fixed	full exclusion	Increased temporal variation in rainfall increases productivity	Photon Flux density	Grassland	Heisler-White et al. (2008)
Fixed	full exclusion	Invasibility ↓	Air temperature	Grass- and shrubland	Kreyling et al. (2008b)
Fixed	full exclusion	Soil biotic processes unaffected	Air temperature	Grassland	Kreyling et al. (2008a)
Fixed	full exclusion	Community productivity unaffected, Tissue die back ↑	Air temperature	Grass- and shrubland	Kreyling et al. (2008c)
Fixed	full exclusion	Flower phenology affected	Air temperature	Grass- and shrubland	Jentsch et al. (2009)
Fixed	full exclusion	Shrub biomass ↑, total plant nitrogen pool ↑	Air temperature	Shrubland	Andresen et al., (2010)
Fixed	full exclusion	Aboveground phytomass ↓, belowground phytomass unaffected	Air & surface temperatures, relative humidity, PAR	Grassland	Shinoda et al. (2010)
Fixed	Full exclusion	Transcription level of genes changes stronger with thermal stress than with water stress	Air temperature	Grassland	Travers et al. (2010)
Fixed	Full exclusion	N_2O emissions unaffected	Air temperature	Shrubland	Carter et al. (2011)
Fixed	Full exclusion	Ecosystem respiration ↓; photosynthesis ↓; plant height ↓	NA	Grassland	De Boeck et al. (2011)

Fixed	Full exclusion	Plant community biomass production unaffected; species specific performance and ecophysiology responds idiosyncratically; biotic interactions affected	NA	Grassland	Jentsch et al. (2011)
Fixed	Full exclusion	Plant survival ↓, Plant growth ↓	NA	Grassland	Buetof et al. (2012)
Fixed	Full exclusion	Plant productivity ↓, plant N ↓, N_2O-emissions ↓	NA	Grassland	Hartmann & Niklaus (2012)
Fixed	Full exclusion	Soil respiration ↓	Air temperature	Shrubland	Selsted et al. (2012)
Fixed	Full exclusion	Low mowing frequency more important for resistance against drought than species richness	Air temperature	Grassland	Vogel et al. (2012)
Fixed	Full exclusion	Biomass ↓; forage quality ↓	Air temperature	Grassland	Walter et al. (2012)
Fixed	Full exclusion	Increased temporal variation in rainfall: changes in phenotype of grass species		Grassland	Avolio & Smith (2013)
Fixed	Full exclusion	Flowering phenology changed	Air temperature	Shrubland	Nagy et al. (2013)
Fixed	Full exclusion	Decomposition ↓, soil biotic activity ↓	Air temperature	Grassland	Walter et al. (2013)
Fixed	Full exclusion	Legume facilitation ↓; N-uptake ↓	NA	Grassland	Arfin Khan et al. (2014)
Fixed	Full exclusion	Forage yield ↓, forage quality ↑	NA	Grassland	Grant et al, (2014)
Fixed	Full exclusion	Grazing affects plant richness and productivity stronger than rainfall variability	NA	Grassland	Koerner et al. (2014)
Fixed	Partial exclusion	Leaf P content ↓, P accumulation in the soil ↑	NA	Forest	Sardans & Penuelas (2004)
Fixed	Partial exclusion	acid and alkaline phosphatase activity ↓, organic extractable P ↑	NA	Forest	Sardans et al. (2008d)
Fixed	Partial exclusion	C and N soil turnover ↓, C and N accumulation in soil ↑	NA	Forest	Sardans et al. (2008c)
Fixed	Partial exclusion	C/N of leaf litter ↓, N availability ↓, enzyme activities ↓	NA	Forest	Sardans & Penuelas (2010)
Fixed	Partial exclusion	Drought can favor exotic species in restoration projects	NA	Grassland	Carter & Blair (2012)
Fixed	Partial exclusion	Plant density during the growing season following drought ↓	NA	Grassland	Carter et al. (2012)
Fixed	Partial exclusion	Heterotrophic respiration ↓	Air temperature	Old field	Suseela et al. (2012)
Fixed	Partial exclusion	Below-ground biomass ↓	NA	Grassland	Fiala et al. (2012)
Fixed	Partial exclusion	Deep root biomass ↑, functional community composition altered	Air temperature, PAR	Old field	Hoeppner & Dukes (2012)

Fixed	Partial exclusion	Photosynthesis ↓, water use efficancy ↑	Air temperature, PAR	Old field	Rodgers et al. (2012)
Fixed	Partial exclusion	Nitrogen mineralization ↓	NA	Old field	Auyeung et al. (2013)
Fixed	Partial exclusion	Tree mortality caused by drought decreases over years of drought exposure	Air temperature, PAR, humidity	Forest	Barbeta et al. (2013)
Fixed	Partial exclusion	Non-structural carbohydrates in trees ↓	Air temperature	Forest	Rosas et al. (2013)
Fixed	Partial exclusion	Soil respiration ↓	Air temperature	Old field	Suseela & Dukes (2013)
Moving	Full exclusion	Stable community composition and biomass production	NA	Grassland	Grime et al. (2000)
Moving	Full exclusion	Site-specific effects on soil respiration (↓↑)	Air temperature	Shrubland	Emmett et al. (2004)
Moving	Full exclusion	Carbon flux ↓	Air temperature	Shrubland	Gorissen et al. (2004)
Moving	Full exclusion	Seedling diversity ↓	Air temperature	Shrubland	Lloret et al. (2004)
Moving	Full exclusion	Leaf relative water content ↓, leaf N concentration ↓, plant composition unaffected	NA	Grassland	Staley et al. (2006)
Moving	Full exclusion	Plant growth ↓, species richness unaffected	Air temperature	Shrubland	Penuelas et al. (2007)
Moving	Full exclusion	Rapid genetic change in shrub species	Air temperature	Shrubland	Jump et al. (2008)
Moving	Full exclusion	Changes in leaf heavy metal concentrations	Air temperature	Shrubland	Sardans et al. (2008a)
Moving	Full exclusion	Leaf N concentrations ↓, stem N concentrations ↑, Stable nitrogen use efficiency, C/N concentration ratio ↑	Air temperature	Shrubland	Sardans et al. (2008b)
Moving	Full exclusion	Shift in species composition (seedlings)	Air temperature	Shrubland	Lloret et al. (2009)
Moving	Full exclusion	Idiosyncratic effects on ecophysiology	Air temperature	Shrubland	Prieto et al. (2009)
Moving	Full exclusion	Leaf litter decomposition ↓, Nitrification rate ↓	Air temperature, PAR, relative humidity	Shrubland	Andresen et al. (2010)
Moving	Full exclusion	Oxidation state of ascorbate ↑, total glutathione ↓	Air temperature	Shrubland	Nogues et al. (2012)
Moving	Full exclusion	Root biomass unaffected	Air temperature	Shrubland	Arndal et al. (2013)
Moving	Full exclusion	No legacy effects on the soil microbial community	Air temperature	Shrubland	Rousk et al. (2013)

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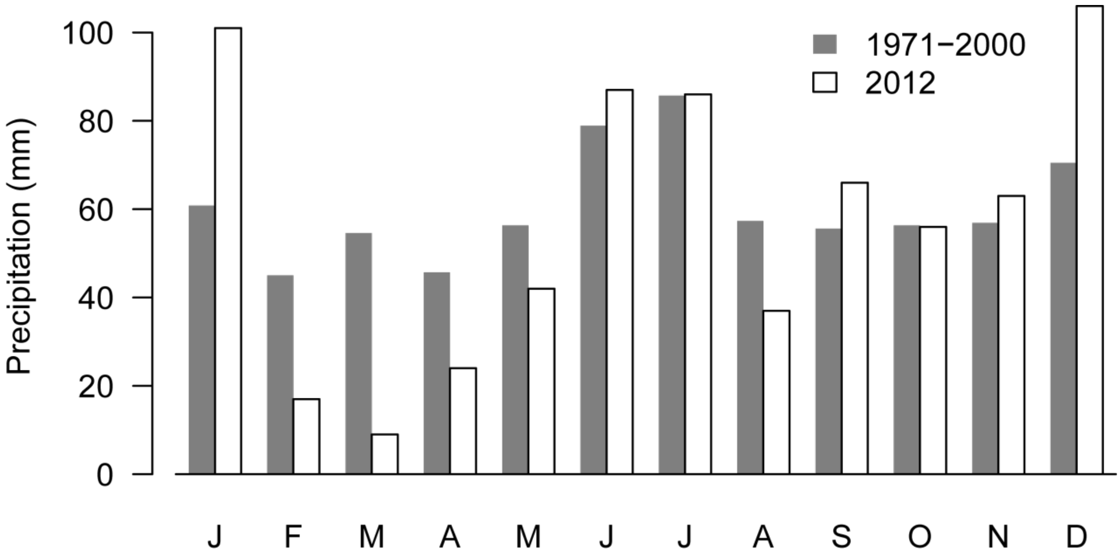
Supporting information S2: Monthly precipitation sums at the study site in the study year 2012 in comparison with the long-term mean conditions.



Supporting information S3: Timing of sampling in the phytometer study in 2012

	Cohort 1	**Cohort 2**	**Cohort 3**	**Cohort 4**	**Cohort 5**	**Cohort 6**
Start of exposure	23.05	30.05	06.06	13.06	20.06	26.06
Stomatal conductance 1st day	23.05	30.05	06.06	13.06	20.06	26.06
Stomatal conductance 2nd day	24.05	31.05	07.06	14.06	21.06	27.06
Stomatal conductance 3rd day	25.05	01.06	08.06	15.06	22.06	28.06
Stomatal conductance 7th day	29.05	05.06	12.06	19.06	26.06	02.07
ΔF/Fm’ of photosystem II	29.05	05.06	12.06	19.06	26.06	02.07
Leaf water potential	†	05.06	12.06	19.06	26.06	02.07

† Due to some instrumental failure, leaf water potential measurements were unsuccessful for 1st cohort.

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Appendix 1: Manuscripts not included in this thesis

Malyshev A, Henry HAL, **Arfin Khan MAS**, Bolte A, Kreyling J. Towards a general understanding of tree bud dormancy: insights from sensitivities to chilling and photoperiod in eight European tree species. In preparation for submission to Agricultural and Forest Meteorology in November 2015.

Steinbauer M, Beierkuhnlein C, **Arfin Khan MAS**, Harter DEV, Irl SDH, Jentsch A, Schweiger AH, Svenning J-C, Dengler J. How to differentiate facilitation from environmentally driven coexistence. Submitted to Methods in Ecology and Evolution in september 2015.

Anik SI and **Arfin Khan MAS**. 2012. Climate change adaptation through local knowledge in the north eastern region of Bangladesh, Mitig Adapt Strateg Glob Change, 17:879-896.

Arfin Khan MAS, Uddin MS and Haque CE. 2012. Rural livelihoods of Rohingya refugees in Bangladesh and their impacts on forests: The case of Teknaf Wildlife Sanctuary In: Uddin N (ed). Narratives on Rohingya Refugees Issue: Re-look at Migration, Security and Integration. ISBN 123-456-78910.

(Eidesstattliche) Versicherungen und Erklärungen

(§ 5 Nr. 4 PromO)

Hiermit erkläre ich, dass keine Tatsachen vorliegen, die mich nach den gesetzlichen Bestimmungen über die Führung akademischer Grade zur Führung eines Doktorgrades unwürdig erscheinen lassen.

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