



Plant growth responses to winter climate change: from among-  
and within-species variation to plant-soil interactions

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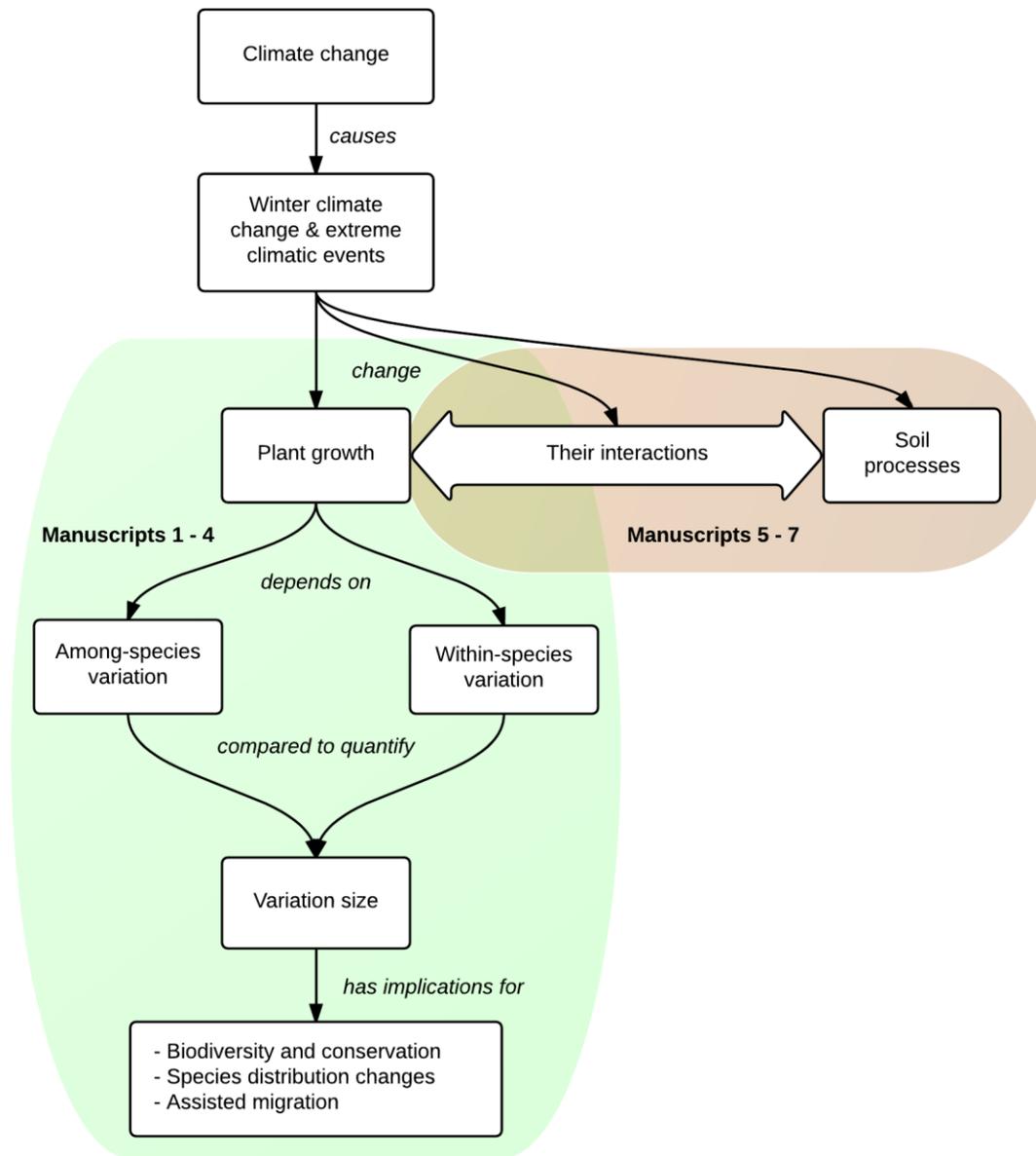
# INTRODUCTION

## Organization of the thesis

This thesis covers response variation in plant growth and plant-soil feedbacks under climate warming during the non-growing season. An overview of the structure is presented in Figure 1. The ongoing climate change is first introduced as an environmental driver modifying plant growth. The discussion is focused on winter climate warming and its projected consequences, which have been experimentally assessed in the included manuscripts to explore variation in plant growth responses under constant warming as well as climate extremes. Winter warming-induced changes in soil processes influencing nutrient supply are also explored, which directly impact plant growth and vice versa.

Among-species and within-species variation in responses to climate change is then introduced, with explanations of the factors driving both variation sources. Implications of direct comparisons between among- and within-species variation in stress responses is subsequently discussed, with key implications for biodiversity and conservation, species distribution changes and assisted migration.

The thesis is concluded with a synthesis of the most important findings, their applicability to improve our understanding of plant growth responses in a warmer world, and their potential for refining general theories in ecology. Emerging questions which need to be answered to bridge current research gaps are stated. Suggestions are finally made for improving experimental designs to be able to better understand how plants respond to climate change across plant organizational levels (ecotypes, species and functional types) and experimental settings.



**Figure 1.** Directional organization of the thesis, showing the key concepts being discussed and the corresponding manuscripts where they are explored in detail.

## **Climate change - an anthropogenic driver of changes in plant growth**

With the recently released IPCC report on climate change, it is now extremely likely that the greenhouse warming effect on the earth is man-made since the 20<sup>th</sup> century (IPCC, 2013). The warming has largely been caused by constantly increasing atmospheric concentrations of greenhouse gases, now the highest since the last 800 000 years. The period from 1983 to 2012 likely marked the warmest 30-year period in the last 1400 years, with a further mean global warming of 2 to 5 °C projected to occur within the next 85 years (IPCC, 2013). Even at the lower estimates of such temperature increase, production of key food crops is projected to be reduced from temperate to tropical regions. Global warming is projected to proceed via multiple major climatic changes. Heat waves, drought, extreme weather events (simulated in **Manuscripts 4 and 5**), a reduction of snow cover in the northern latitudes and the accompanying increased soil temperature variability (simulated in **Manuscript 6**) or a complete absence of soil frost (simulated in **Manuscript 7**), and rising sea level are some of the global changes very likely to occur over the 21st century, affecting plant growth and subsequently economies and ecosystems with threats to food security and agricultural incomes (IPCC, 2013).

### **A focus on winter warming**

Winter climate change effects on plant growth are understudied in comparison with summer warming effects (Kreyling, 2010). Impacts of winter warming are more complex than summer warming (Makoto, 2014) because in addition to temperature increases, changes in snow cover impact plants via multiple factors, including insulation changes and water availability (Groffman *et al.*, 2001a), still holding many unanswered questions to plant responses (Rapacz *et al.*, 2014). Warming effects can even be opposite in the summer and winter, which can reverse the plant species order in terms of most susceptible species to warming. During summer warming for example, lichen species suffer more compared to vascular plants, having been linked to a worldwide lichen decline (van Wijk, *et al.*, 2004), but are more tolerant of sudden mid-winter extreme warming events (Bjerke *et al.*, 2011).

### *Stronger temperature increases over winter*

Particularly strong temperature increases have occurred and are projected to continue occurring over winter, especially at high latitudes (Sætersdal *et al.*, 1998; Hay & McCabe, 2010). Highest temperature increases are predicted to occur in the arctic regions, likely to experience mean increases of 8.3°C warming under the highest emissions scenarios by the end of the century (IPCC, 2013). The temperature increases are also not occurring regularly over the entire year. In the last 50 years winter temperatures in Alaska have increased almost twice that of the annual average temperature increase, with some agricultural regions experiencing average temperature increases as high as 7°C in the last 30 years (Karl *et al.*, 2009). Regarding temperature variability, it is unclear if higher or lower variability will occur in the future (Rapacz *et al.*, 2014), but sudden extreme frosts will continue to occur (IPCC, 2013). Warming in the arctic can also enhance the frequency and magnitude of weather extremes in mid latitudes (Semenov, 2012).

### *Effects of strong winter temperature increase*

More than half of the land area in the Northern Hemisphere is seasonally frozen (Zhang *et al.*, 2003). Between 1979 and 2011 snow cover in the northern hemisphere has declined by 17.8% per decade (Derksen & Brown, 2012) and continues to decline, with a further 7% to 25% reduction projected by the end of the 21<sup>st</sup> century (IPCC, 2013). This is of crucial importance, because a 10-20 cm snow layer maintains most soil temperatures in the northern latitudes close to zero °C with little temperature fluctuation (Thorsen & Höglind, 2010). Furthermore, a snow layer of 30-40 cm can decouple soil and air temperatures (Edwards *et al.*, 2007). Reduced soil insulation can paradoxically lead to colder soil temperatures over winter in a warmer world (Venalainen *et al.*, 2001). The strong temperature increase will also reduce permafrost between 37% and 81% by the end of the 21<sup>st</sup> century (IPCC, 2013).

Snow influences plant phenology, growth and species composition. Earlier snowmelt can result in reduced plant growth and changes in community composition despite an extended growing season. The primary cause of this occurrence is frost sensitive species not being able withstanding the sudden frost events in the spring (Wipf *et al.*, 2006). Both increased and decreased soil freezing can occur with decreased snow cover, depending on mean air

temperatures in a specific region (Hardy *et al.*, 2001; Henry, 2008; Kreyling & Henry, 2011). Nonetheless, experimental evidence projects that increased soil freezing will occur over large areas of the temperate zone (Durán *et al.*, 2014b).

### **Plant responses to warmer winters**

A recent review on growth of herbaceous plants in warmer winters concluded that no concluding evidence exists on overriding plant responses (Rapacz *et al.*, 2014). Plants are likely to respond in phenology shifts (Menzel & Estrella, 2001), lengthening of the growing season (Keeling *et al.*, 1996), changes in species ranges (Walther, 2001), species distribution (Pauli *et al.*, 1996) and species abundance (Smith, 1994), as well as changes in plant growth rates (Graybill & Idso, 1993; Bisgrove & Hadley, 2002). Snow mold damage is also directly linked to the duration of snow cover (Gaudet *et al.*, 1989). Species-specific tolerances to the respective climate stresses and differences in competitive ability can change community structure and composition. This can take place as some species take better advantage of warmer temperatures and an extended growing season, shifting species distributions and causing extinction of some species (Hughes, 2000; Kreyling *et al.*, 2010). Interactions between community composition and winter warming induced nutrient cycling changes are described in **Manuscript 5**.

### *Changes in plant cold acclimation*

Cold acclimation is a suite of changes in gene expression and physiology that increases plant tolerance to cold temperatures (Kalberer *et al.*, 2006). A reduced photoperiod and declining temperatures initiate the start of cold acclimation in perennial plants (Stout & Hall, 1989; Thomashow, 1999). In autumn, soluble sugars accumulate from starch mobilization (Sauter *et al.*, 1996) and, together with other solutes, lower the freezing point of the intracellular solution (Poirier *et al.*, 2010). Acclimation is completed at low and sub-freezing temperatures with the synthesis of anti-freeze and dehydrin proteins and structural changes in membrane lipids (Kozłowski & Pallardy, 1997). With global warming, temperatures stay warm longer in the fall, causing delays in plant senescence (Menzel *et al.*, 2006; Ibanez *et al.*, 2010), leaving plants less time for cold acclimation (Rapacz *et al.*, 2014). An important factor in how plants respond to

warmer fall depends on their relative dependence on photoperiod as a key cold acclimation cue. Increased clouding and initiation of cold acclimation at lower day lengths can decrease cold acclimation capacity, especially for photoperiod-sensitive species. In addition, increased soil water content due to melting snow and possible subsequent ice encasement, increased atmospheric CO<sub>2</sub> concentration, and cold deacclimation (reduction of cold hardiness) following warm spells all interact in determining plants' ability to cold acclimate and withstand damage through frost and ice encasement (Rapacz *et al.*, 2014). More research is needed on minimum light requirements for adequate cold acclimation in the fall (Rapacz *et al.*, 2014). **Manuscript 4** reviews techniques well suited to quantify cold acclimation.

### *Midwinter warm spells*

Warming is projected to occur in pulse events in addition to gradual warming (IPCC, 2013). Such events have only recently been studied in experimental manipulations (Bokhorst *et al.*, 2008; Kudo, 2014; Schuerings *et al.*, 2014). The influence of short term highly variable climatic events on plant responses is underrepresented in winter climate warming research (Kreyling, 2010). Modelling plant growth responses under climate change, such as grassland yields in temperate regions, often does not incorporate winter survival (Rapacz *et al.*, 2014). Despite warmer winter temperatures however, plant winter damage and mortality may not decrease due to winter warming events causing anoxia (Dalmannsdottir *et al.*, 2012), ice encasement and enhanced plant freeze damage (Bokhorst *et al.*, 2009).

Periods of warm temperatures occurring during the non-growing season can cause plants to lose the acquired cold acclimation, resulting in premature cold deacclimation and growth. During deacclimation, metabolites responsible for the production of freeze-retardant compounds such as stress proteins are catabolized and genes responsible for the producing of such metabolites during cold acclimation are down-regulated (Kalberer *et al.*, 2006). Photosynthetic activity also increases at the expense of frost tolerance as cryoprotective carbohydrates are respired (**Manuscript 5**). Consequently, the risk of freezing injuries in winter and spring could rise (Pagter & Arora, 2013). Cold spells can last from hours to days and are known to have caused food shortage and famines in the past centuries by killing crops prior to harvest

(Semenov, 2012). Considerable damage to mid-winter warm spells in arctic vegetation has been observed, both in manipulated and in naturally occurring warm spells, with up to 87% summer reduction of growth in a dwarf shrub (Bokhorst *et al.*, 2009). Mid-winter warming effects on frost hardiness is inconclusive however, with unclear consequences of ice lens formation and increased possibility of pathogen attacks (Rapacz *et al.*, 2014). There is less knowledge on deacclimation in herbaceous plants compared to woody plants (Pagter & Arora, 2013). Therefore, deacclimation resistance and reacclimation capacity needs to therefore be studied more closely, with special focus on real field conditions and variations among and within species (Rapacz *et al.*, 2014). In **Manuscript 4** the effects of midwinter warm spells of variable duration on common grassland species are presented. In **Manuscript 5** physiological plant responses are quantified as plants are released from their dormant state during a simulated mid-winter thaw event.

Sudden midwinter warm spells particularly contribute to soil temperature fluctuations, leading to an increased frequency of soil freeze thaw cycles (FTC) (Henry, 2007b), although this effect depends on the current mean regional air temperatures, causing region-specific increases or decreases in the number of experienced FTC (Henry, 2008; Kreyling & Henry, 2011). **Manuscript 7** looks at potential ecosystem responses of carbon cycling to midwinter warming, comparing the increased carbon loss due to higher soil respiration and enhanced plant productivity due to uptake of the mobilized nitrogen (N). Nutrient cycling is also addressed more in depth in **Manuscript 6**, focusing on the effects of colder soils in the warmer world (Groffman *et al.*, 2001a). The occurrence of FTC and subsequent plant damage is also shown to depend on the prevailing air temperatures, with different FTC dynamics and subsequent plant responses occurring at different experimental sites.

#### *Advancement of the growing season*

Spring is advancing with the earlier onset of warmer temperatures (Parmesan & Yohe, 2003; Ibanez *et al.*, 2010). The mean tree growing season has advanced by 7 days per degree of warming in Europe (Chmielewski & Rotzer, 2001) and is projected to advance by 1.5 months for forage crops in Canada within the next 50 years (Belanger *et al.*, 2006). The observed growing

season changes have long-term influences on carbon storage and vegetation cover (Linderholm, 2006). Photoperiod changes are stable throughout the year, unlike temperature fluctuations, which are unpredictable. Photoperiod sensitivity is therefore of crucial importance in terms of determining plant responses to warming temperatures. Larger reliance on photoperiod cues for the timing of growth initiation can limit the advance of the growing season with advancing spring. The observed advances in budburst have occurred at different rates in tree species (Heide, 1993; Wang *et al.*, 2014) and have complex effects on plant-pollinator interactions due to species-specific pollinators not being able to follow changes in budburst phenology (Bale & Hayward, 2010). Budburst will likely also not keep advancing in a linear relationship with warmer winter and spring temperatures due to interactions with chilling and photoperiod (Körner, 2007). Key factors primarily responsible for tree budburst and their generality across species are described in detail in **Manuscript 1**.

#### *Plant strategies to minimize and prevent frost damage*

Strong temperature fluctuations occur daily, monthly and annually in the temperate zone, whereas photoperiod varies seasonally, with higher variation at high latitudes. *Fagus sylvatica* is a good example of a remarkable adaptation to changing temperate seasons. The species experiences 320 freeze-free days in its southern distribution and 166 freeze-free days in the north (Vitasse *et al.*, 2014). Despite such differences in the growing season length, the ecotypes of *F. sylvatica* have adapted to synchronize their growth patterns without a higher incidence of frost damage with higher latitude or elevation (Vitasse *et al.*, 2014). Within-species variation in *F. sylvatica* and its distinctly different winter dormancy pattern is explored in detail in **Manuscript 1**. In general, temperate tree species survive freezing temperatures by escaping, avoiding and tolerating frost (Levitt, 1972; Körner & Riedl, 2012). Shedding of leaves removes the most sensitive tissues, escaping frost. Timing for budburst to occur outside of the frost-prone time period as well as using super cooling with the help of anti-nucleating agents to prevent liquid from freezing inside plant tissues also enhance frost tolerance (Kuwabara *et al.*, 2013). Tree species potentially at a higher risk of frost damage following mid-winter warming are the ones achieving low mid-winter dormancy depth, making it easier for their dormancy to be broken

(Pagter & Arora, 2013). **Manuscript 1** quantifies the mid-winter dormancy levels of eight European tree species.

**Running summary:** the earth is currently undergoing accelerating climate warming, which is resulting in multifaceted environmental changes. The understudied winter warming is of particular interest due to stronger temperature increases and complex interactions among snow cover, soil temperature, plant cold acclimation and deacclimation, frost damage and changes to the growing season. The mentioned factors all affect how plant growth responds to winter warming, yet the picture is far from complete. The medium in direct contact with plants, the soil, is undergoing changes too and understanding of plant-soil interactions is essential to more realistically quantify plant growth changes.

### **Soil processes and plant-soil interactions**

Linking above- and below-ground climate change responses is essential in understanding terrestrial ecosystem responses to winter climate change, especially because interactions between plant species and biogeochemical cycles are largely unknown (Makoto *et al.*, 2014). Plant community changes interact with ecosystem processes by modifying the amount and quality of carbon input into the soil, in turn modifying above and below ground processes (Bardgett, 2011). There is extensive literature on biogeochemical responses to winter climate change. As this thesis is mainly on plant responses, soil chemistry changes are only briefly mentioned, with a focus on plant soil interactions. Only nutrient availability changes with direct and most influential plant influences are discussed. **Manuscripts 5, 6 and 7** are focused on these plant-soil interactions.

A primary factor that influences ecosystem responses to warming is the transfer of carbon from roots to the soil, regulating carbon cycling and sequestration. Net primary productivity influences the quantity and composition of soil organic matter (litter and rhizodeposits, microbial biomass and soil fauna), which is also directly modified through warming and extreme weather events. Plant growth is in turn directly modified through changes in nutrient cycles, which arise from changes in soil organic matter (Bardgett, 2011). Primarily changes in carbon (C) and N cycles due to winter warming have been studied in this thesis (N in **Manuscripts 5 and 6**, and C

and N in **Manuscript 7**), which are particularly sensitive to soil freezing (Matzner & Borken, 2008). Winter climate regulates fluxes and sources of C and N leached during snowmelt, affecting the ecosystem budgets and water quality (Campbell et al., 2014a). **Manuscripts 5, 6** and **7** address N leaching in detail.

### *N importance for plant growth*

Carbon and N play a central role in many ecosystem processes (Tateno & Chapin, 1997). N compounds are involved in incorporation of C into plant structure via photosynthesis, with C making up about half of plant biomass (Vitousek, 1982). Northern temperate ecosystems are experiencing increasing rates of atmospheric N deposition (Galloway *et al.*, 2004) and the amount of added N that is retained in the ecosystem will influence primary productivity and plant species composition (Tilman & Downing, 1994). Seasonal water availability and temperature changes are central to the influence of N pools and their flows between plants, microbes, soil, air and water (Vitousek *et al.*, 1997; Shibata *et al.*, 2013). Climate warming can increase rates of microbial litter decomposition and N mineralization, in turn increasing plant productivity (Sierra, 1997; Rustad *et al.*, 2001). N cycling can also be altered by winter warming through changes in soil freezing dynamics (Henry, 2008). Decomposition, mineralization and nitrification of N compounds from frost-killed fine roots, disruption of soil aggregates (Larsen *et al.*, 2002) and lysis of microbial cells (Yanai *et al.*, 2004) are the proposed mechanisms for increased soluble N supply following FTC (Fitzhugh *et al.*, 2001; Henry, 2007a). N can then be lost through leaching and N<sub>2</sub>O emissions as a result of reduced plant N uptake over winter (Sharma et al., 2006; Matzner & Borken, 2008), leading to ecosystem N losses (Henry, 2007a). N uptake, as affected primarily by freezing and community composition, is quantified in **Manuscripts 5, 6** and **7**.

### *Seasonal plant N uptake*

Winter microbial communities remain active in temperate regions by virtue of the insulative property of the snowpack (Brooks *et al.*, 1998). Inorganic nutrients accumulate under snow because net N mineralization continues and plant uptake is low (Brooks *et al.*, 1998). FTC activity can also contribute to high winter N availability through microbial lysis (Henry &

Jefferies, 2002). As a result, in temperate and subarctic regions, soil N generally peaks in midwinter (Henry & Jefferies, 2002; Schmidt & Lipson, 2004).

Uptake of soluble N is slowed when plants are dormant in winter (Laine *et al.*, 1994). Nonetheless, winter N uptake may be maintained by vascular plants remaining physiologically active and maintaining photosynthesis at subzero air and soil temperatures (Larsen *et al.*, 2007). Some graminoids can take up N in situ over winter (Andresen & Michelsen, 2005) in quantities comparable to summer N uptake in similar grassland species (Nasholm *et al.*, 2000; Bardgett *et al.*, 2003). Root damage by ice encasement (Ouellet, 1976) can decrease winter N uptake, as shown in trees found in hardwood forests (Tierney *et al.*, 2001; Weih & Karlsson, 2002).

Although belowground biological activity is maintained under the snowpack in the winter, it remains low until soil temperature reaches and surpasses 4°C (Groffman *et al.*, 2012). The increased mineralization of organic matter provides readily available nutrients which can be taken up by plants in the early spring (Muller & Bormann, 1976; Zak *et al.*, 1990). Whether advances in plant phenology will match an earlier onset of microbial activity is not known (Polgar & Primack, 2011) and is an important factor influencing plant responses to warmer winter and spring temperatures. Evidence hints at a widening gap (Groffman *et al.*, 2012) and highlights the need to consider plant-soil interactions more closely, since the nutrient cycling directly affects plant growth and vice versa.

#### *FTC effects on nutrient cycling and plant growth*

In regions where reduced snow cover is expected, increased soil temperature variability is also likely due to the diminished insulation. More frequent soil temperature changes, often accompanied by increased frequency of freeze-thaw events can lead to N losses from soils as leachate (as also found in **Manuscripts 5, 6 and 7**), following microbial and plant damage, (Fitzhugh *et al.*, 2001; Groffman *et al.*, 2001b; Tierney *et al.*, 2001). This occurs because of increased inorganic N entering the soil solution via increased microbial activity, converting organic N from dead tissue (Durán *et al.*, 2014a). Higher N runoff into streams can lead to coastal and freshwater eutrophication, as well as a decrease in ecosystem biodiversity through acidification (Vitousek *et al.*, 1997; Galloway *et al.*, 2004). N leaching is enhanced due to

reduced root nitrogen uptake, as shown both in grasses (Malyshev & Henry, 2012a) and in trees (Campbell *et al.*, 2014b). In a temperate system, there is also evidence that winter N uptake can improve summer growth, at least in graminoids (Kreyling *et al.*, 2008; Malyshev & Henry, 2012b).

FTC can lead to significant increases in nitrate, phosphate, and base cation losses (Fitzhugh *et al.*, 2001; Cleavitt *et al.*, 2008), which appear to be driven by increases in the root mortality of some tree species, such as *Acer saccharum* (Tierney *et al.*, 2001; Cleavitt *et al.*, 2008). However, the response to soil-freezing events varies, that is, they are not always marked by increases in nitrate losses (Hentschel *et al.*, 2009). This variation in nitrate loss may be driven by variation in the response of dissolved organic C dynamics to soil freezing. In some cases, soil freezing mobilizes dissolved organic carbon, which stimulates immobilization or denitrification, which, in turn, prevents a nitrogen response (Groffman *et al.*, 2012). On the other hand this may be beneficial for water systems, due to reduced inorganic N input through N leaching (Durán *et al.*, 2014a).

Species abundances have been found to differ in FTC manipulated plots vs. non FTC manipulated plots, showing that community composition follows different trajectories and are not limited to the immediate growing season, lasting over many years showing contrasting year to year effects (differences disappearing after the first year). Long-term changes to ecosystems are possible though short-term mid-winter occurrences, with more diverse communities having shown to be more stable (in maintaining species abundances) after FTC disturbances (Kreyling *et al.*, 2011). Community composition has also been shown to play a large role in determining the timing of FTC effects, with a grassland community being more responsive in the first year and a heath community being more responsive in the second year (Kreyling *et al.*, 2010). Effects of prolonged periods of warming with an absence of soil frost are described in **Manuscripts 5** and **7**, while **Manuscript 6** addresses how FTC occurrence is modified by site- specific microclimate.

**Running summary:** changes to soil nutrient cycles are likely to have the most noticeable effect on plant growth with winter warming. Nitrogen is one of the primary nutrients that plants acquire from the growing medium and its deficiency most commonly leads to growth reductions.

Plant N uptake in winter, although reduced, is important not only influencing N loss from the soil as leachate, but also in determining plant growth in the growing season. Increased soil temperature fluctuations can lead to FTC and play a major role in soil N cycling and plant N uptake. Having discussed how winter warming affects plant growth overall and how changes in soil processes modify plant growth via plant-soil interactions, the variation in plant responses to these abiotic and biotic interactions is introduced below.

## **Variation in plant responses to environmental change**

### **Development of plant variation**

Variation in plant responses to environmental change starts at the individual plant level. Despite this plant-to-plant variation, populations of plants exposed to regional environmental conditions often develop more uniform responses from other geographically environmentally separated populations. This occurs due to genetic differentiation that originates from physical barriers, preventing cross-breeding among populations (Königer *et al.*, 2012; Bradburd *et al.*, 2013). Normally, the greater the geographical separation between populations, the greater is also the genetic divergence (Bradburd *et al.*, 2013). Disproportionally large divergence may also take place within short distances however, due to environmental heterogeneity or dispersal limitations resulting in limited gene flow (Wright, 1943; Edelaar & Bolnick, 2012). Such genetically distinct populations, adapted to specific environmental conditions develop through selection, are called ecotypes. Many terms have evolved to describe genetically distinct populations within a species, such as provenance, accession, variety, cultivar, strain, population etc. For this thesis, the term ecotype is always used when climatic adaptation is evident in a population, and the term provenance is used for geographically separated populations for which local adaptation has not yet been detected. The pathways to speciation are multifold and complex (Lexer & Widmer, 2008; Schluter, 2009; Soltis & Soltis, 2009), but in essence a species is the final step in ecotype divergence, where interbreeding is no longer possible.

## **Among-species variation**

It is clear that species differ in many ways from morphology to resource use, from stress tolerance to competitive ability and in responses to environmental disturbances. Species-specific responses to drought, warming during summer and winter as well as spring and winter frost (**Manuscript 4**), photoperiod and winter chilling requirements (**Manuscript 1**) and plant N composition and uptake (**Manuscripts 4 ,5 and 6**) were found. Rather than simply documenting species-specific differences, finding generalities among responses in certain species groups is more beneficial in projecting patterns of plant responses to climate change. Current trends are summarized below.

In tree studies, differences in temperature and photoperiod sensitivities have shown to be the driving factors behind species-specific responses to the extension of the growing season (Way & Montgomery, 2014). Deciduous trees have shown more sensitivity towards increasing temperatures and are thus projected to increase their growth rates faster than coniferous species, with temperature limited northern trees showing predominantly positive temperature responses and southern trees being inhibited (Way & Oren, 2010). Tree species with less photoperiod sensitivity are more likely to migrate north and adapt to warmer temperature with less influence of the photoperiod limitations (Way & Montgomery, 2014). Relative spring and fall photoperiod sensitivity also differs among species, making clear projections based on individual studies problematic in determining the overall tree response to warming with respect to phenology, with fall senescence delays contributing more to growing season changes than spring advances in parts of Europe (Fracheboud *et al.*, 2009). In **Manuscript 1**, relationships between temperature and photoperiod sensitivity and winter dormancy are explored. Coniferous and deciduous trees with different life strategies are also ranked according to potential sensitivity to winter and spring warming. Across plant functional groups (grasses, forbs and woody plants), higher temperature influence compared to photoperiod is common (Campbell *et al.*, 2007). **Manuscript 2** also concludes that grasses and trees may be similar in cold acclimation differences across the latitudinal distribution of species.

Responding to higher temperatures with increased growth can be a dangerous strategy in case of sudden frost occurrence. Physiology changes have been shown to be strongly species-specific after simulated prolonged mid-winter warming in the arctic on dwarf shrub species

(Bjerke et al., 2011). There are also species such as winter rye (*Secale cereale*) however, which are able to grow and cold acclimate at the same time, (Griffith & McIntyre, 1993), which may have an advantage over species not exhibiting this dual nature. In **Manuscript 4** a midwinter warm spell and a subsequent frost are simulated to relate the length of warming to the acquired frost damage. The resulting among-species variation is then quantified.

### **Within-species variation**

Charles Darwin wrote that organism “forms” which show a “character” of the same species but are not grouped under the same species by naturalists are “most important to us” (Darwin, 1859). Generally, an adaptation is a phenotypic feature which is functionally designed by past natural selection, and which improves Darwinian fitness relative to alternative features (Williams, 2008). The development of ecotypes is always a tug of war process between natural selection and gene flow. The development of distinct ecotypes and the relative divergence from ecotypes is enhanced by strong local selection pressures and is countered by gene flow between the populations, which homogenizes the developing differences (Aitken & Whitlock, 2013). Gene flow is influenced by geography, population size and environmental gradients (Kawecki & Ebert, 2004).

There is a rich literature in within-species adaptations to their environment. As shown in the literature review (Table 1), within-species variation exists virtually in every trait and in response to every environmental gradient or stress factor, just as in different species. The strength of local adaptation has recently been displayed, whereby even in fish, with their much faster mating and gene exchange rates compared with plants, increased gene flow was not as influential as local adaptation in causing phenotypic changes. Specifically, introduced fish populations with distinct phenotypes did not influence the phenotype of a local population, but showed a change in own phenotype, resembling the local phenotype after 12 generations (Fitzpatrick *et al.*, 2014). Therefore, local adaptation maintains and contributes to the evolution of genetic differences, therefore contributing to the maintenance of genetic variation (Hedrick *et al.*, 1976; Hedrick, 1986).

**Table 1:** Literature review of studies comparing among- vs. within-species variation, 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 28<sup>th</sup> 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 partitioned into among and within-species variation and subsequently compared. Shading differentiates among studies where variation was dominant within species (white), among species (dark grey) or approximately equal (light grey).

Type of study	Species	Presence of induced stress	Parameters	Dominant variation	Reference
Field drought and warming experiment	two grass species; two populations per species from Mediterranean and temperate origins.	under stress	biomass, nitrogen nutrition, survival	within-species (“much greater”)	Poirier <i>et al.</i> , 2012
Natural gradient sampling	two tree species; 27 tree populations per species (30 km apart)	without stress	leaf chemical composition, N resorption, carbon isotope discrimination, S LA, lifespan	within species (2 to 3 times greater)	Walters & Gerlach, 2013
Field winter survival experiment	Three legume species; 10 or more cultivars per species from southern and western or central Europe	without stress	winter survival	within-species (“much greater”)	Annicchiarico & Iannucci, 2007
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	Fichtler & Worbes, 2012
Meta-analysis (observational studies)	various	without stress	functional traits (leaf mass : area, N content)	within - species / equal	Read <i>et al.</i> , 2014

Type of study	Species	Presence of induced stress	Parameters	Dominant variation	Reference
Glasshouse experiment simulating winter and tropical growth conditions	four tree species and one to five populations per species (US, Mexico, Costa Rica)	under stress	growth rate, freeze tolerance	among - species (2 to 6 times greater)	Koehler <i>et al.</i> , 2012
Global database	129 alien species	without stress	plant functional traits (height, biomass, SLA)	among - species	Ordóñez, 2014
Matrix population models from literature data	50 perennial plant species; multiple populations ( $\geq 2$ ; $\geq 1$ km apart) and multiple matrices per population	without stress	population growth rate	among - species	Buckley <i>et al.</i> , 2010
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 greater)	He <i>et al.</i> , 2010
Review paper	C3 species	without stress	photosynthetic capacity	among - species	Kouki, 2010
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 (at least 3 times greater), except for SLA.	Kichenin <i>et al.</i> , 2013

Type of study	Species	Presence of induced stress	Parameters	Dominant variation	Reference
Wood elemental analysis	nine tree species, one to five populations per species	without stress	physical and chemical wood characteristics	among -species	Pande <i>et al.</i> , 2007
Environmental gradient sampling	13 common plant species	without stress	five functional traits	among -species (~2.5 times greater)	Albert <i>et al.</i> , 2010
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	Wardle <i>et al.</i> , 2009
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	Long <i>et al.</i> , 2011
Geographical gradient	three carnivorous 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	Dominguez <i>et al.</i> , 2014

The deacclimation effect of warmer temperatures on lowered frost tolerance during mid-winter (**Manuscript 4**) and spring (**Manuscript 4, Manuscript 5**), as well as warming effects on cold acclimation (**Manuscript 2**) and dormancy loss over winter and spring (**Manuscript 1**) have

been documented. Furthermore, within-species response variation to summer drought has also been quantified in **Manuscript 4**, where warming and drought effects were simulated. Ecotypes differed in their responses in all of the aforementioned temperature, photoperiod and rainfall manipulations, with the exception of tree provenance responses to chilling and light sensitivity treatments, where a uniform response was found across northern Europe for *F. sylvatica* provenances (**Manuscript 1**). Therefore, strong support was found for within-species variation in different plant functional groups (grasses, trees) and for a variety of parameters, showing that local adaptation can shape different traits, the latter being in turn selected for under different environmental conditions.

Plant ecotypes have specific adaptations that can determine their unique responses to winter climate change. Increased frost tolerance of northern plant ecotypes (for example in grasses) may not always be an advantage (Rapacz *et al.*, 2014). In acquiring the additional frost tolerance, northern ecotypes tend to experience an earlier growth reduction due to higher sensitivity to short photoperiods (**Manuscript 2**; Rapacz *et al.*, 2014). In **Manuscript 2** northern and southern ecotypes of the common grass *Arrhenatherum elatius* are compared in their cold acclimation strategies, showing that southern *Arrhenatherum elatius* ecotypes are less sensitive to photoperiod and may benefit from warmer fall by increasing their biomass longer into the fall. With warmer fall temperatures, southern ecotypes may respond more positively, provided the acquired frost tolerance is enough for the random fall frost events. Such north to south latitudinal pattern in photoperiod sensitivity has also been shown in trees (Junttila *et al.*, 2003). Due to available literature on within-species differences in cold acclimation, setting up well-planned experiments to identify best-adapted ecotypes to a modified (warmer temperatures occurring during short late fall temperatures) and warmer acclimating season in the future is becoming increasingly important.

Development of within-species variation via adaptation marks the beginning of the speciation process, and is based on the premise that populations become better adapted to their local environment through natural selection (Hereford, 2009). However, the advantage of the local ecotype does not always hold true, with every third study, published up to and including 2005, showing no better performance of a local ecotype in a transplant, compared to non-native populations (Hereford, 2009). A Meta-analysis has also showed that plant life history, spatial or

temporal habitat heterogeneity, and geographic scale did not influence the extent of local adaptation (Leimu & Fischer, 2008). Other more influential drivers of local adaptation may include insufficient environmental differentiation, limited gene flow and genetic drift (Hereford, 2009). With the three factors being equal however, it is not known if species-specific abilities exist in adapting to new environments quicker, thereby leading to the creation of ecotypes quicker and subsequently increasing the chances of being able to adapt to climate change through greater overall genetic variation. Results from this thesis show support however, that such ability may be species-specific, with not all species from the same origins displaying similar local ecotypic adaptations (**Manuscript 4**; Beierkuhnlein *et al.*, 2011; Kreyling *et al.*, 2012).

In short, it has thus been shown that among- and within-species variation is present in virtually all phases of plant growth cessation and growth resumption. Dormancy induction, cold acclimation, deacclimation, reacclimation, and dormancy loss all vary at the species and sub-species level largely due to relative influence of light and temperature sensitivities and their interactions (Olsen, 2014). The among- and within-species temperature and photoperiod sensitivities at the growth initiation face of trees are addressed in **Manuscript 1** while within-species differences in the same factors for growth cessations in a common grass are discussed in **Manuscript 2**. Nonetheless, generalities do exist, such as higher heat sum requirements being correlated with shorter periods of chilling (Junttila & Hanninen, 2012). A further generality has been discovered within the scope of this thesis; higher mid-winter dormancy being correlated with faster rates of decrease in dormancy depth (**Manuscript 1**). Discovering such generalities is important in being able to classify the most sensitive species and ecotypes to climate change.

### **Comparing among- and within-species variation**

It is clear that both among- and within-species variation has been well documented (Table 1). Almost all such studies however, have focused on measurement of plant traits under stress-free conditions. Most commonly among- vs. within-species comparisons aimed to quantify the predominant source of variation in plant chemical compositions, physiological parameters and plant functional traits (Table 1). Both among- and within-species variation was found to predominate in individual studies, depending on the species, environmental gradient and

measured parameters. The main purpose of most of these studies was actually not to compare the two sources of variation. Instead, plant characteristics were searched for, which best correlate with environmental variation to better explain what environmental drivers are most influential in explaining variation in plant traits. A specific comparison between among- and within-species variation is important, however, for general theories describing biodiversity (described in detail in the synthesis section below) which stand to benefit from the inclusion of within-species components. Furthermore, species distribution models, which commonly assume homogenous responses within a species, can be refined by the inclusion of within species variation. **Manuscript 4** presents a novel among- vs. within-species comparison under stress, whereby extreme climatic events were simulated to generate maximum variations in plant responses, testing the extent of variation at two basic plant organizational levels (species and ecotype level). The finding that within-species variation under stress is not lower than among-species variation validates a closer examination of the driving forces behind local adaptation.

**Running summary:** Plant variation in growth responses, which is evident from the individual level to plant functional types, shows the difficulty of projecting plant responses to winter climate change. A comparison of among- and within-species variation under stress reveals that within-species variation can be as high as among-species variation. In the synthesis that follows, generalities are described, that despite the species-, ecotype- and experimental site-specific differences, can be made with respect to plant growth responses to climate warming. Subsequently, implications of high within-species variation are discussed.

## **Synthesis: towards a more complete understanding of plant growth responses to climate change**

### **Temperature and photoperiod as drivers of among- and within-species variation**

Multiple aspects of winter warming were simulated on different species from different functional groups, as well as on different ecotypes of different grass species. Among- and within-species differences were found, as plants' responses varied with respect to cold acclimation, cold

deacclimation, loss of dormancy and frost tolerance. Overall, temperature and photoperiod sensitivity played a major role in generating among- and within-species variation.

Photoperiod was found to play an especially important role during the cessation and resumption of growth. A lower influence of photoperiod on specific tree species during winter dormancy loss (**Manuscript 1**) is likely result in a more closely-matched tracking of winter and spring warming. Likewise, lower photoperiod sensitivity of southern grass ecotypes, which matches the pattern in southern tree ecotypes, may also respond with faster growth increase per degree of warming than the northern ecotypes (**Manuscript 2**). Temperature also played an important role, allowing southern ecotypes to achieve higher biomass than northern ecotypes (**Manuscript 2**). Temperature also influenced plant growth rates and frost damage due to different deacclimation rates during midwinter warming in common grassland species (**Manuscripts 4 and 5**). Pulsed warming resulted in modified soil nutrient cycling based on plant community composition and species-specific N uptake (**Manuscript 6**) and biomass responses (Schuerings *et al.*, 2014). Continuous warming over two winters led to plant community-specific changes in elemental composition compared to no warming (**Manuscript 7**), while extreme midwinter warming also caused species- and community-specific N uptake and biomass responses (**Manuscript 5**).

### **Practical implications of high within-species variation**

Very few studies have measured among-species variation against within- species variation. Part of the reason is that different ecotypes are intuitively always found much farther away from each other compared to different species growing in a single community. The assumption then arises that for a given region experiencing an environmental change, among-species differences dominate within-species differences. This assumption however, may not necessarily hold true. Firstly, although environment heterogeneity and local adaptation is normally suggested to increase at larger geographic scale (Galloway & Fenster, 2000), plant ecotypes are able to evolve over very small distances due to reduced gene flow compared with animals. Secondly, as shown in two of our experiments, testing frost and warming as well as drought tolerance (**Manuscript 4**), two communities can contain the same species, yet the responses of the two communities can

differ in the species response ranking. This can occur when one species is dominant in one community, but loses out to its neighbor in a distant community due to ecotypic differences. Ecotypes within different species may therefore adapt to different climatic conditions at different rates and / or extent. Thirdly, assisted migration can bring distant ecotypes and result in mixtures, although with unknown outcomes and persistence of the genetic differences (**Manuscript 4**). In our results, within-species variation was not lower than variation among species from different functional groups (**Manuscript 4**). Such findings have implications for assisting plants with adaptation to climate change (explained below) and modelling plant distribution changes (explained in **Manuscript 4**).

### *Implications for assisted migration*

Plants are generally expanding their ranges northward and to higher elevations (Chen *et al.*, 2011; Lazarus & McGill, 2014). The rate of migration can be reduced by the occurrence of unpredictable cold periods (Jalili *et al.*, 2010). Much less certainty exists about species' trailing edge impacts (Hampe & Petit, 2005), with specialized montane species experiencing range contractions due to narrow, specialized niches (Hodd *et al.*, 2014). The rate of these geographical shifts is occurring slower than the rates of warming in most landscapes, increasing the threat of range contraction or even extinction (IPCC 2013). Assisted migration is defined as the intentional anthropogenic movement of individuals and populations (Aitken & Whitlock, 2013). By introducing the species outside of its current range a species, an earlier opportunity is provided to develop adaptations to the changing climate. Such practice has come under criticism however, due to unpredictable and potentially damaging effects on the ecosystems where the introduction has taken place (Webber *et al.*, 2011; van der Putten, 2012). Within the range of a species, ecotypes better adapted to anticipated climate change for a specific region may be found in a distant location from the less adapted ecotype. The translocation of the better adapted ecotypes results in assisted gene flow, which is much less likely to have undesirable and unexpected effects, potentially arising with species introductions (Aitken & Whitlock, 2013). Nonetheless, outbreeding depression is possible, which is a reduction in offspring fitness relative to parental types following hybridization between populations (Aitken & Whitlock, 2013). Determining the extent of local adaptation within a species, that is the number of distinct climatically adapted

ecotypes, is thus useful in identifying suitable species for which ecotype mixing through assisted migration is advised (Aitken & Whitlock, 2013). Trees especially have extensive genetic variation, which could allow adaptation to climate change through natural selection but dispersal limitations hinder tree migration (Savolainen *et al.*, 2007). Assisted migration could alleviate this problem.

#### *Using within-species variation in plant breeding*

The extensive within-species variation can be utilized not only through assisted migration but also in breeding novel ecotypes, resistant to temperature stress associated with climate change. Among- and within-species differences in cold acclimation, deacclimation and reacclimation are determined by non-correlated traits, which promotes the development of genetically distinct populations suited to specific local environmental conditions (Kalberer *et al.*, 2006). Genomic selection holds promise for development of better adapted forage crops (Heffner *et al.*, 2009). However, great difficulty exists in predicting plant ideotypes (plants with model characteristics enabling reliable growth prediction (Donald, 1968) with the highest winter-hardiness in the future (Rapacz *et al.*, 2014). Genetic manipulation of transcription factors and regulators involved in low temperature and cold acclimation can be used to improve winter survival and decrease winter damage (Rapacz *et al.*, 2014), although side effects include reduced growth, later flowering and reduced seed production (Yamaguchi-Shinozaki & Shinozaki, 2001; Morran *et al.*, 2011).

#### *Implications for conservation and biodiversity preservation*

Plants are more likely to undergo sympatric speciation due to a higher probability of self-pollination. It is therefore more likely that distinct ecotypes being brought into close proximity to each other will remain as distinct ecotypes and contribute to the overall increase in ecotypic diversity in a given area. Genetic mixing among populations brought into close proximity is a two-way process. The advantage is in increasing fitness through preventing inbreeding depression (Tallmon *et al.*, 2004), while the disadvantage is through limiting population

divergence, thus preventing populations from achieving peak adaptation and reducing their fitness (Garcia-Ramos & Kirkpatrick, 1997). Population mixing is relevant for conservation of biodiversity, as species have been rescued from extinction through population mixing (Johnson *et al.*, 2010). On the other hand, some species are negatively impacted via the introduction of new populations, resulting in introgression of invasive alleles (Muhlfeld *et al.*, 2009). The central question that has emerged from our direct among- vs within-species comparisons is how much and how quickly gene population mixing through assisted migration disrupts the maintenance of unique local adaptation within ecotypes (**Manuscript 4**). Very few studies are available addressing this question, which may hold answers to adaptation potential of plant populations to climate change.

**Running summary:** Generalities across plant organizational units and site-specific plant-soil interactions were found within the scope of this thesis. Key drivers of among- and within-species variation were temperature and photoperiod. Within-species variation, being as high as among-species variation under stress has the potential to enhance the ability of species to adapt to climate change faster. Additional use of the high within-species variation includes modification of biodiversity theories and improved plant distribution modelling. To conclude the introduction of the thesis, the limits of species-specific studies are summarized before the questions and experiments are stated, which should be answered and implemented in the future to better understand plant growth changes.

## **Emerging questions**

It is challenging to predict plant responses in a warmer world, primarily because of complex interactions between direct plant responses to winter warming and indirect response pathways arising through plant-soil interactions (Mori *et al.*, 2014). Plants, microbes and soil organisms all affect nutrient cycling (Makoto *et al.*, 2014), the latter in turn feeding back to impact plant growth. Target species, functional groups, winter conditions, habitat and the type of climate change all influence climate change responses and therefore make results focusing on one factor limiting in their implications (Makoto *et al.*, 2014). Many climate change studies have

focused on species-level responses related to phenology, physiology and distribution changes. Species-level research is not sufficient however, to gain a complete understanding of climate change effects because of interactions between species and community processes (Mori *et al.*, 2014). This thesis combined diverse experiments on individual plant responses from different plant organizational levels and explored indirect plant-soil feedback mechanisms. New questions have emerged that should stimulate future experimental design aimed at providing more general conclusions, applicable across greater scales of specificity (e.g., not being organism / site / season specific).

*Theme 1: Can we arrive at generalities across species-specific climate warming responses and plant-soil interactions?*

Question 1. What easily measurable plant traits can predict more complex plant characteristics (such as cold acclimation, deacclimation and photoperiod sensitivity), which are directly related to responses to winter climate change?

Reason: the simpler it is to measure a plant's ability to respond to climate change, the more plants can be measured and the more general conclusions can be drawn about changes in species compositions in the future.

Question 2. Do plants respond uniformly from different plant functional groups as long as they share similarities in traits mentioned in Question (1)?

Reason: **Manuscript 4** has shown that the specific ecotypes present in a region may be as important as the species growing there in determining their responses. Certain traits may likewise be influential in determining plant sensitivity to environmental stress, allowing to make generalizations across plant functional groups.

Question 3. Can complexity of plant-soil interactions be reduced, determining the most influential and significant feedbacks affecting plant responses?

Reason: **Manuscripts 5, 6 and 7** have shown that plant community composition, field site and soil freeze dynamics all interact to bring about variable results with respect to changes in nutrient cycling and subsequent plant growth responses. Experiments are needed to quantify the relative influence of each of the mentioned factors to be able to more conclusively project plant responses under projected winter warming scenarios.

*Theme 2: What questions need to be answered to apply the knowledge of high within-species variation?*

Question 4. What environmental factors determine the rate and extent of within-species variation?

Reason: ecotypic differentiation may be species-specific, whereby under the same environmental gradient some species develop more locally adapted ecotypes than other species. Discovering factors that allow within-species variation to increase faster in certain species can help to advance the development of ecotypes better suited to future climate.

Question 5. What is the value of using ecotype mixtures in preserving and enhancing plant biodiversity?

Reason: even though the potential value of within-species variation has been shown in **Manuscript 4**, such an idea needs to be tested in practice, combining different ecotype and species mixtures under stress to determine most stress tolerant and resistant plant mixtures.

Question 6. Can high phenotypic plasticity compensate for a lack of species and ecotypic diversity in helping to adapt to climate change?

Reason: phenotypic plasticity is the capacity of a single genotype to produce different phenotypes in response to varying environmental conditions (Witman & Agrawal, 2009), and was not explicitly quantified for species and ecotypes in this thesis but should be mentioned as a useful tool for projecting the ability of ecotypes and species to rapidly adapt to climate change. Adaptive plasticity can improve survival with environmental change (Chevin & Lande, 2010).

Most responses to climate change result from phenotypic plasticity and not new adaptations, according to one meta-analysis (Gienapp *et al.*, 2008). It is therefore important to measure phenotypic plasticity and use it as a primary tool for the selection of ecotypes for assisted migration and for determining species that are in need of assisted migration the most (ones with the least phenotypic plasticity).

### **Suggestions for future among- and within-species experiments**

#### *Specific reflections on experimental design from the thesis*

Reflection 1. When more frequent temporal and spatial sampling is better than increased replication.

In **Manuscript 1** it was shown that a loss of replication in favour of finer temporal resolution can be beneficial for determining rates of change and the types of relationships (such as linear or exponential) between plant responses and environmental parameters. A more frequent sampling approach is also better suited at detecting thresholds or tipping points in a system under stress.

Reflection 2. Benefits of carrying out controlled experiments under natural / semi-natural conditions to simulate extreme climatic events.

A simulation of warming events or trends will never replicate what occurs or will occur in nature. More controlled experiments are generally better suited for answering questions related to the mechanism driving a particular plant response while in situ climate change experimental designs are better suited to quantify realistic plant responses without necessarily explaining the mechanisms driving these responses (**Manuscript 3**). Combinations of both experimental designs were used within the scope of this thesis that combine realism and controlled simulation of environmental extreme events. Cooling trucks administered frost to plants deacclimating at ambient conditions (**Manuscript 4**), climate chambers were used to stimulate budburst to tree seedlings overwintering in the field (**Manuscript 1**) and heating wires and lamps simulated midwinter warming in situ (**Manuscripts 5,6 and 7**). In all mentioned cases controlled selection

and plant arrangement in plots likely reduced variability which would have occurred in completely natural vegetation, while allowing natural deacclimation and overwintering conditions to take place. Efficient and timely measurement of responses to spontaneous and rare extreme events is a hit-and-miss strategy with big time lags. Nonetheless, preparedness can be improved by having apparatus, personnel and protocols ready to respond rapidly when extreme events do occur. Meta analyses focused on experimental design are also useful in finding generalities or pointing out discrepancies in results due to experimental design that can be avoided with future designs.

### Reflection 3. Reduced experiment complexity to simplify interpretation of results.

Another double-edged sword is the number of factors used in an experiment. Too few factors may not address factor interactions while inclusion of too many factors may make interpretation of results too complicated. In this thesis, a cautious approach was taken with relatively few factors (2-3) compared to other larger climate change experiments (i.e. The Jasper Ridge global change experiment (Shaw *et al.*, 2002) and multifactor experiments with CO<sub>2</sub>, warming and drought manipulations (Mikkelsen *et al.*, 2008). The number of factors therefore depends on the size of the experiment. Large scale and longer lasting experiments benefit from higher factorial designs at the beginning of the experiment due to more complicated incorporation of factors later on. Smaller scale experiments with a short time frame and fewer replicates can benefit from lower factorial designs, being easier to repeat and / or replicate in a different setting / location.

### *Improvements and additions to experimental design for the future*

#### Suggestion 1. Further collaboration and cooperation among scientists.

In an age of ever-increasing connectivity, collaboration and cooperation among biogeochemists, ecologists, modelers and geneticists is becoming easier and should be taken advantage of. For example, genomics, transcriptomics and proteomics together describe how the hereditary code is transcribed and used to assemble the structural units of plants. Geneticists working with biologists and ecologists can explain the root causes of differences in responses to climate change among- and within-species. Likewise, modelers can impose species- and ecotype-

specific responses on a climatic map, projecting large scale plant growth responses. A cooperation with Stefan Michalski from the Helmholtz Centre for Environmental Research was started as part of an experiment on plasticity in grass ecotypes along a latitudinal gradient. Genetic differences and plasticity of plant traits are currently being compared (manuscript in preparation).

Suggestion 3. Experiments covering both growing and non-growing seasons / lasting several years.

Warming over one spring may have carry over effects in the following spring with respect to phenology (Fu *et al.*, 2014). Warming studies across seasons accounting for legacy effects are important (Way & Montgomery, 2014). Response time lags, increased stress tolerance to recurrent extreme events (Walter *et al.*, 2013), reduced seed germination or differential fitness of offspring can be masked by short term plant responses. Long term monitoring and studies are therefore suggested to describe more conclusively the changes that occur with climate change and their causes (Groffman *et al.*, 2012). **Manuscript 1** has focused on the transition from the dormant to the growing season in trees **Manuscript 2** looked at cold acclimation differences, which happen in the spring. What counts however is the total sum of climate effects over the course of at least one complete growth cycle to conclude species and ecotype response rankings.

Long-term studies also mean that more robust experiments have to be set up, where plant measurements could be made easily and without time constraints. This way long-term measurements are easier to maintain even when with personnel changes. In such cases, potted experiments are limited and mesocosms with parameters not requiring time consuming measurements are favored. More multiple year experiments are essential as long-term winter climate change studies total only 4 % of all cases (Kreyling, 2010).

Suggestion 4. Use of simultaneous multisite experiments allows for clearer and novel conclusions.

A new trend in experiments is underway worldwide that aims to address the issue of multiple factors which make conclusions from site specific experiment results problematic. The

recently suggested coordinated distributed experiments (Fraser *et al.*, 2013) are a good example of such a design where simple and low cost experimental design is favored to implement replicated experiments in a variety of environmental settings , spanning multiple ecosystems. The multisite design allows to separate sampling processes from the effects of biotic interactions, arriving at conclusions which can be generalized across diverse environments. Also the emergence of new patterns only possible with multisite approach such as species richness vs. plant biomass relationship being stronger in communities not as dominated by a single species (Hector *et al.*, 2002).

#### Suggestion 5. Including biomass as a central measured parameter

Biomass was the most ecotype-specific parameter, which varied not only overall among ecotypes, but also was affected differently in different ecotypes under different environmental disturbances / manipulations. Biomass is a very common parameter measured in plants because it encompasses the total sum of multiple parameter changes such as nutrient uptake, height, growth rate, changes in physiology, etc., to bring a net effect on plant growth performance. The drawback of measuring such a parameter is that no precise mechanistic explanation can usually be given for the respective changes in biomass. It can be compared to a black box analysis, where the true cause of reduced growth is unknown. Thus, biomass measurements are likely to most accurately detect the overall positive or negative effect of a treatment, but do not pinpoint a true cause of the decrease or increase. It is therefore advisable to measure biomass and specific parameters expected to be responsible for the biomass changes.

## Summary of manuscripts presented in the thesis

**Table 2.** As shown in Figure 1, Manuscripts 1 to 4 explore among- and within-species variation to winter climate change, while Manuscripts 5 to 7 help clarify the complexity of plant-soil interactions. Below the generalities and unique findings are compared among the 7 Manuscripts.

Specific question(s) addressed	Key finding	Conclusion / Implication(s)
1. Among and within-species temperature and light sensitivity during loss of winter dormancy	Species-specific dormancy loss patterns and a general trend was found, relating dormancy depth and its rate of decrease	Species-specific extension of growing season is likely. Species can be ranked according to dormancy loss patterns, relating to potential responses to an earlier spring.
2. Within-species variation in temperature and light sensitivity during cold acclimation	Southern grass ecotypes achieved higher biomass after a mild frost due to less temperature and photoperiod sensitivities	Within a species, strong differences in responses to warmer fall and winter may occur.
3. Suggested methodology to detect within-species cold acclimation differences	Step-wise method to determine cold acclimation differences	The described method can be used to determine the level of within-species variation
4. Among- vs. within-species variation in plant responses under climatic extremes	Within-species variation matched among-species variation	Within-species variation should be included in biodiversity theories and species distribution models
5. Effects of prolonged winter warming and increased temperature variability on N leaching, as influenced by climate and community composition	Grasses were likely less damaged than shrubs and took up more N leading to less N leaching. Colder site likely resulted in more plant damage leading to more N leaching	Warming spells and not just FTC can lead to N leaching, which is modified by species / functional type-specific N uptake capabilities
6. Effects of increased temperature variability on N leaching, as influenced by climate and community composition	Decomposition and soil N content increased with higher temperature variability. Specie-specific plant N uptake was lower at the colder upland site.	Warming spells and not just FTC can lead to N leaching, which is modified by species / functional type-specific N uptake capabilities
7. Effects of long term soil heating (2 seasons) on soil respiration, soil and plant-soil N cycling	Increased soil respiration and no increase in soil N, potentially due to higher plant N uptake, fast N leaching or loss of N in gaseous form through nitrification and denitrification	Increased soil respiration reinforces positive feedback of warming, especially in southern temperate sites where the chance of sudden frost is lower

## **Declaration of own contribution**

Experimental design refers to contribution to the conceptual design and set up of each experiment.

Data collection refers to the physical tasks required to collect data for each experiment

Data analysis refers to using software to sort / make calculations / analyze the collected data in each experiment.

Writing refers to formulating of sentences and paragraphs as well as inserting fitting references

Visuals refers to the creation of tables and figures used in the manuscripts.

Literature review refers to performing a search through web of science for a specific topic with the result being a table with a list of references.

Discussion refers to development of new ideas and explanations, which are incorporated into a manuscript based on the results.

### **Manuscript 1: Towards a general understanding of tree bud dormancy: insights from sensitivities to chilling and photoperiod in eight European tree species**

Authors: Andrey Malyshev, Hugh A.L. Henry, Mohammed A. S. Arfin Khan, Andreas Bolte Juergen Kreyling.

Status: in preparation

Journal: Tree Physiology

Own contribution: experimental design: 90%; data collection: 70%; data analysis: 90%; writing: 75%; visuals: 50 %; discussion: 75 %

### **Manuscript 2: Relative effects of temperature vs. photoperiod on growth and cold acclimation of northern and southern ecotypes of the grass *Arrhenatherum elatius***

Authors: Andrey Malyshev, Hugh A.L. Henry, Juergen Kreyling.

Status: Published

Journal: Environmental and Experimental Botany

Own contribution: experimental design: 80%; data collection: 70%; data analysis: 90%; writing: 75%; visuals: 75 %; discussion: 75 %

**Manuscript 3: Common garden experiments to characterize cold acclimation responses in plants from different climatic regions**

Authors: Andrey Malyshev, Hugh A.L. Henry, Juergen Kreyling.

Status: Published

Journal: Methods in Molecular Biology

Own contribution: literature study; writing: 75%; visuals: 90 %; discussion: 50 %

**Manuscript 4: Plant Responses to climatic extremes: within-species variation equals among-species variation**

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

Status: Submitted

Journal: Global Change Biology

Own contribution: experimental design: 30%; data collection: 40%; data analysis: 50%; writing: 50%; literature review: 70%; visuals: 50 %; discussion: 50 %

**Manuscript 5: Nitrogen leaching is enhanced after a winter warm spell and controlled by plant community composition in temperate zone mesocosms**

Authors: Juergen Kreyling, Jan Schuerings, Andrey V. Malyshev, Lukas Vogt, Christiane Werner, Anke Jentsch.

Status: in preparation

Journal:

Own contribution: data collection: 25%; writing: 10% visuals: 15%; data analysis 15%; discussion: 5%

**Manuscript 6: Increased winter soil temperature variability enhances nitrogen cycling and soil biotic activity in temperate heathland and grassland mesocosms**

Authors: J. Schuerings, A. Jentsch, V. Hammerl, K. Lenz, H. A. L. Henry, A. V. Malyshev, J. Kreyling

Status: Published

Journal: Biogeosciences

Own contribution: data collection: 15%; writing: 10%; visuals: 15 %; discussion: 20 %

**Manuscript 7: Absence of soil frost affects plant-soil interactions in temperate grasslands**

Authors: Jan Schuerings, Carl Beierkuhnlein, Kerstin Grant, Anke Jentsch, Andrey Malyshev, Josep Peñuelas, Jordi Sardans & Juergen Kreyling

Status: Published

Journal: Plant and Soil

Own contribution: data collection: 15%; writing: 10%; visuals: 15 %

**Table 3.** Noteworthy personal scientific engagements predominantly during the 3-year period of this doctoral work (01.2012 – 01. 2015).

Type of engagement	Description	Ongoing tasks
Proposal writing	Co-writing of the DFG proposal, which formed the basis for experiments in Manuscripts 1 to 4	
Experimental design and implementation	Developing of optimal experimental design for winter warming experiment in Manuscript 4 and both experiments leading to Manuscripts 1 and 2	
Additional cooperation	Co-designing and carrying out an experiment quantifying plasticity and genetic differences in a grass along its latitudinal distribution limits (Stefan Michalski, Helmholtz Centre for Environmental Research).	Submission of manuscript
Student supervision 1	Co-designing and carrying out a climate chamber freeze thaw cycle experiment for a masters Student (Charlotte Dietrich) on influence of climatic adaptation of <i>Dactylis glomerata</i> ecotypes originating from 12 countries	Submission of manuscript
Student supervision 2	Co-supervising 2 Bachelors students with their Bachelors´ theses on exploring factors which explain species and ecotype-specific tree chilling requirements	
Presentation of results at five international conferences	Ecological Society of America annual meeting (2 times – 2011, 2013) GfOe Annual Meeting (2 times – 2012, 2014) British Ecological Society Annual meeting (2014)	

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# Manuscript 1: Towards a general understanding of tree bud dormancy: insights from sensitivities to chilling and photoperiod in eight European tree species

Journal: Tree Physiology (to be submitted)

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## Abstract

Budburst responses to winter and spring warming influence productivity and can cause shifts in tree species composition in response to climate warming. To improve projections of tree phenological changes, a more complete understanding of variation in relationships between chilling and forcing temperature requirements and photoperiod sensitivity is needed to explain species-specific bud dormancy controls. We investigated budburst responses to chilling and photoperiod at high temporal resolution from mid-winter to early spring in seedlings of eight coniferous and deciduous temperate trees, ranging from pioneer to late successional species. Provenances of *F. sylvatica*, a dominant European species with deep dormancy and high photoperiod sensitivity were also included, enabling the testing of generality in bud dormancy patterns within species. Tree seedlings were over-wintered in a common garden and transferred weekly into climate chambers at forcing temperatures from December to April. Budburst was observed under 16 and 8 hour photoperiods. Firstly the general trend was examined across all tree species. Species requiring longer forcing periods (associated with deeper dormancy) in mid-winter had faster rates of decrease in forcing temperature requirements. Secondly, 5 levels of budburst sensitivity were examined among the 8 species, based on midwinter dormancy depth, rate of loss of dormancy depth and photoperiod sensitivity. Budburst dates under ambient field

conditions were consistent with the species rankings. Overall, our results indicate a possible adaptation in trees with deep dormancy (at both the species and sub-species level) to reduce growth delays in spring by breaking dormancy rapidly. We also rank diverse European species in terms of potential sensitivity to midwinter and spring warming, and discuss the potential implications of species compositional changes.

**Keywords:** tree seedling, dormancy depth, day length, ecotype, intraspecific variation, interspecific variation, winter climate change.

## **Introduction**

In Europe, the growing season has advanced on average by 11 days from the 1960s to the 21<sup>st</sup> century, mostly due to earlier leaf emergence (Linderholm, 2006, Menzel *et al.*, 2006). Timing of spring growth plays a vital role in influencing biomass production by modifying the growing period, with bud burst dates influencing carbon assimilation and the tree energy budget (Kindermann *et al.*, 1996). For example, a 20% extension in the growing season can increase annual net ecosystem productivity of a deciduous forest by as much as 50% (Dragoni *et al.*, 2011). However, earlier leaf flushing also represents a tradeoff between earlier photosynthesis and an increased risk of frost damage (Gömöry and Paule, 2011; Mimura and Aitken, 2010).

Photoperiod and temperature largely determine the latitudinal distribution of tree species (Thomas and Vince-Prue, 1996), and they are the most important factors controlling phenology in dominant tree species outside the tropics (Morison and Morecroft, 2008). Relative budburst sensitivity to photoperiod is of particular importance for assessing tree growth responses to climate warming (Heide, 1993a; Schaber and Badeck, 2003; Vitasse *et al.*, 2009). Photoperiod can limit tree sensitivity to warmer winter and spring temperatures, the latter of which has been widely shown to cause species-specific advances in the onset of spring growth (Cleland *et al.*, 2007; Laube *et al.*, 2014; Menzel *et al.*, 2006; Menzel and Estrella, 2001; Willis *et al.*, 2008). Phenological models can also have increased predictive power when they use both temperature

and photoperiod as explanatory parameters for budburst (Cannell and Smith, 1983; Chuine and Cour, 1999).

In temperate deciduous trees, dormancy or “the inability of a bud to burst at normal growth temperatures in long days” (Sogaard *et al.*, 2008), is generally released by a required chilling period, optimally at chilling temperatures near 5 °C (Myking and Heide, 1995; Perry, 1971) with the duration varying among (Farmer, 1968) and within northern deciduous species (Heide, 1993b). Subsequent accumulation of forcing temperatures (> 5°C) (Bailey and Harrington, 2006) then leads to budburst in trees released from dormancy (Kramer, 1994). Short photoperiods can prevent premature dormancy release when the chance of frost may still be high (Häkkinen *et al.*, 1998; Heide, 1993b) while long photoperiods can also compensate for insufficient winter chilling temperatures, reducing budburst sensitivity to warmer temperatures (Häkkinen *et al.*, 1998; Heide, 1993a; Sanz-Pérez *et al.*, 2009).

The interplay between chilling requirements, spring forcing temperatures, and photoperiod in influencing bud burst is controversial (Vitasse *et al.*, 2009) with longer photoperiod and longer exposure to chilling temperatures reducing the thermal time to budburst in some species (Falusi and Calamassi, 1990; Heide, 1993b), but not having an effect in others (Heide, 1993b, Schaber and Badeck, 2003). With respect to chilling requirements, even within evergreen species they can vary greatly; *P. abies* needs 4 weeks of chilling at 3 – 6 °C (Dormling *et al.*, 1968) while *Pinus monticola* needs 16 weeks of chilling at the same temperature (Steinhoff and Hoff, 1972; Wells *et al.*, 1979). Generally, early successional or pioneer species have been suggested to have low chilling and forcing requirements, and they often have low photoperiod sensitivity (Basler and Koerner, 2012; Koerner and Basler, 2010; Laube *et al.*, 2014). However, a recent literature review suggested that no clear characterization exists for classifying photoperiod-sensitivity in trees (Way and Montgomery, 2014).

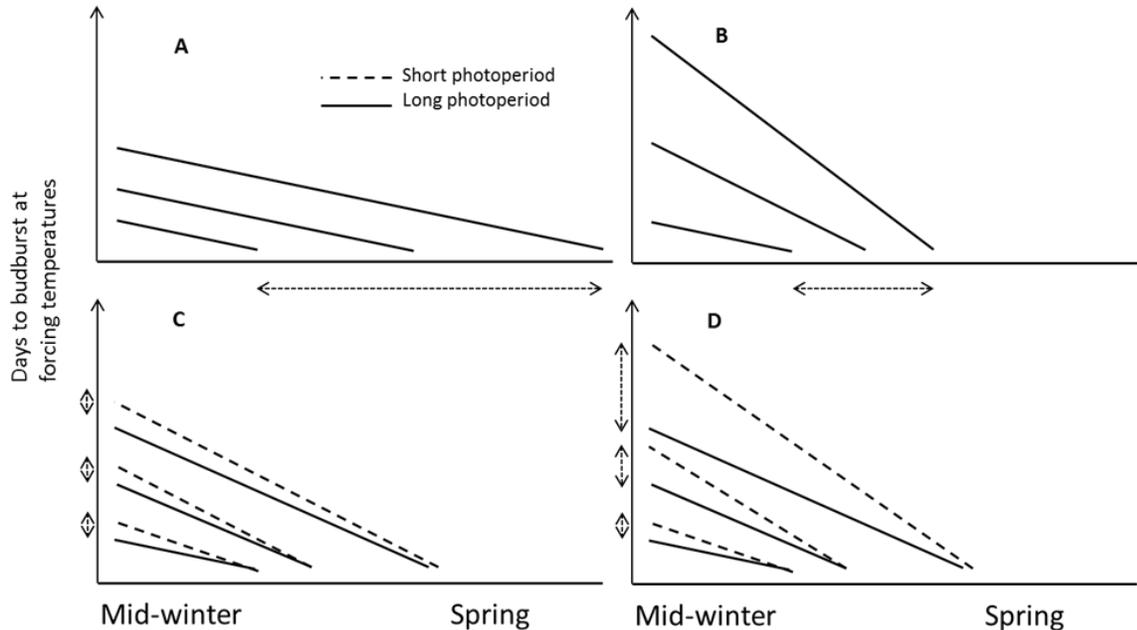
Longitudinally-, latitudinally- and altitudinally- specific phenological differences among populations have also been documented (Chmura and Rozkowski, 2002; Wuehlisch *et al.*, 1995) with within-species differences in bud phenology being genetically driven (Campbell *et al.*, 1989; Ekberg *et al.*, 1991). Such genetic control enables budburst order in seedlings to be maintained

across years with varying temperatures down to seed family level (LI and Adams, 1993), although environmental effects have been shown to explain much more variation in budburst dates compared to genetic differences (Vitasse *et al.*, 2013).

Establishing more universal relationships between factors controlling tree bud-burst is needed to help classify species according to their phenotypic sensitivities in response to climate warming. Certain trends have been documented, such as the decrease in thermal time to leaf-out with increased chilling (Caffarra and Donnelly, 2011; Heide, 1993a; Murray *et al.*, 1989) and possibly higher photoperiod sensitivity in winter compared to spring at low accumulation of chilling temperature (Heide, 1993a; Myking and Heide, 1995b). Furthermore, species achieving deeper dormancy and having higher chilling requirements in mid-winter do not always burst bud later in the spring due to faster rates of decrease in forcing requirements (Heide, 1993a; Laube *et al.*, 2014). The nature of these relationships (linear vs. non-linear) as well as their generality across species have not been explored in detail. To our knowledge, no study has addressed bud dormancy depth, as influenced by chilling sum and photoperiod, and simultaneously quantified among and within-species responses at fine temporal resolution.

In the current study, we compared chilling temperature and photoperiod sensitivities among and within species. We used days to bud break under forcing conditions, which is a proxy for dormancy level (Li *et al.*, 2005) as our central response parameter. We compared budburst sensitivity changes from mid-winter to spring, examining the relative influences of accumulated chilling hours and simulated variable photoperiods in 8 European tree species known to differ in their dormancy patterns (Way and Montgomery, 2014). We also explored within-species variation in *F. sylvatica*, one of the most photoperiod sensitive ((Fu *et al.*, 2012; Kramer, 1994) and dominant tree species in Europe (Vitasse and Basler, 2013). Among and within species budburst patterns were determined from mid-winter to spring at fine temporal resolution (7-10 day intervals), allowing us to detect changes in budburst sensitivities before and during the onset of the tree growing season. We predicted that both across species and across ecotypes of *F. sylvatica* (1) deep mid-winter dormancy (a higher number of days to budburst at forcing temperatures) results in delays in spring budburst, which are minimized via fast rates of loss in dormancy depth (Scenario B in Fig. 1), (2) photoperiod sensitivity is higher in mid-winter than in

spring, and is positively correlated with deep midwinter dormancy (Scenario D in Fig. 1), and (3) spring budburst can be explained using the combined knowledge of species' midwinter dormancy depth and its loss rate as well as photoperiod sensitivity.



**Figure 1.** Influence of mid-winter dormancy depth and photoperiod on the rate of dormancy depth loss. **Panels A and B:** possible adaptation of species with deeper mid-winter dormancy / higher chilling requirements. A – no adaptation: All species have similar rates of decrease in dormancy resulting in the species with deeper mid-winter dormancy minimizing their dormancy and bursting bud much later in spring. B – with adaptation: Species with deeper mid-winter dormancy have faster rates of decrease in forcing temperature requirements, minimizing budburst delays in spring. **Panels C and D:** Possible explanation behind higher photoperiod sensitivity in temperature-sensitive tree species. C: Mid-winter dormancy depth / higher chilling requirements have no relationship with photoperiod sensitivity. D: Higher photoperiod sensitivity positively influences dormancy depth attained in mid-winter.

## Materials and Methods

We selected eight *Fagus sylvatica* provenances from seed sources in Northern France, Northern Germany and Poland to represent the variation in photoperiod sensitivity among provenances differing in winter climate and chilling days in their native origins (Table 1). The trees were cultivated from seed in greenhouses at the Thünen-Institute, Germany (Institute of Forest Genetics, Institute of Forest Ecosystems), with the exception of one provenance (Table 1 - Germany 3), which was cultivated together with the other tree species as described in the

following paragraph. In late fall 2012, the two-year old plants were potted in 2 L pots (Hermann Meyer KG), and in the summer of 2013 they were delivered to the Bayreuth Ecological Botanical Garden and placed under a rainout shelter constructed of a steel frame (GlasMetall Riemer GmbH) and covered with a polyethylene sheet (0.2mm, SPR5, Hermann Meyer GmbH) permitting 90% of photosynthetic radiation. The seedlings were kept under the shelters until fall, and received 1 litre of water per week per pot. The substrate used was made up from Ah and Bv soil horizons of the forest soil in Eberswalde, Germany, made up predominantly of 87.2% sand, 9.8% silt, 2.9% clay and >0.5% humus.

**Table 1.** Geographical and climate characteristics of seed origins of *Fagus sylvatica* L. used in the experiment, representing the within-species diversity category. Climate data were obtained from worldclim (Hijmans *et al.*, 2005), using a resolution of 10 arc-seconds.

Country	Latitude °N	Longitude °E	Altitude (m) a.s.l.	Mean minimum temperature of coldest month (°C)	Mean maximum temperature of warmest month (°C)	Annual mean temperature (°C)
France	50.25	1.88	<200	1.2	21.6	10.3
Germany 1	52.95	8.35	<200	-2.3	21.4	9.0
Germany 2	53.40	9.83	<200	-3.2	22.0	9.2
Germany 3	50.04	11.85	800-920	-5.7	19.8	5.9
Poland 1	52.68	17.67	<200	-6.2	23.5	8.2
Poland 2	53.08	18.93	<200	-6.3	23.6	7.8
Poland 3	53.27	19.50	<200	-7.2	23.3	7.8
Poland 4	53.02	19.60	<200	-7.7	23.5	7.8

Other tree species (*Abies alba* Mill., *Picea abies* (L.) H.Karst., *Quercus robur* L., *Acer pseudoplatanus* L., *Sorbus torminalis* (L.) Crantz, *Tilia cordata* Mill., *Larix decidua* Mill.) were grown from local German seed sources (Table 2) in a tree nursery in the vicinity of Bayreuth, Germany (Bayerische Staatsforsten AöR - Pflanzgarten-Stützpunkt Bindlach) and delivered to the Ecological Botanical Garden in Bayreuth at the end of October 2013. All provenances and tree species were transplanted into 8 cm × 8 cm × 20 cm deep pots at the end of October using the same soil used to grow the *F. sylvatica* provenances.

**Table 2.** Geographical and climate characteristics of seed origins of tree seedling species used in the experiment, and the respective tree seedling ages at the start of experiment. Climate data were obtained from worldclim (Hijmans *et al.*, 2005), using a resolution of 10 arc-seconds.

Species	Latitude °N	Longitude °E	Elevation	Sowing date	age(years)
<i>Acer pseudoplatanus</i> L.	49.89	11.05	409-537	2012 (April)	2
<i>Picea abies</i> L.	49.90	10.50	445-450	2010 (May)	4
<i>Fagus sylvatica</i> L.	50.04	11.85	800-920	2013 (May)	1
<i>Abies alba</i> Mill.	49.96	11.04	325-379	2009 (October)	4
<i>Sorbus torminalis</i>	49.84	10.38	270	2011 (June)	2
<i>Larix decidua</i> Mill.	50.08	9.25	440	2012 (May)	2
<i>Tilia cordata</i> Mill.	49.45	11.14	330	2011 (June)	2
<i>Quercus robur</i> L.	49.52	11.06	307-311	2013 (May)	1

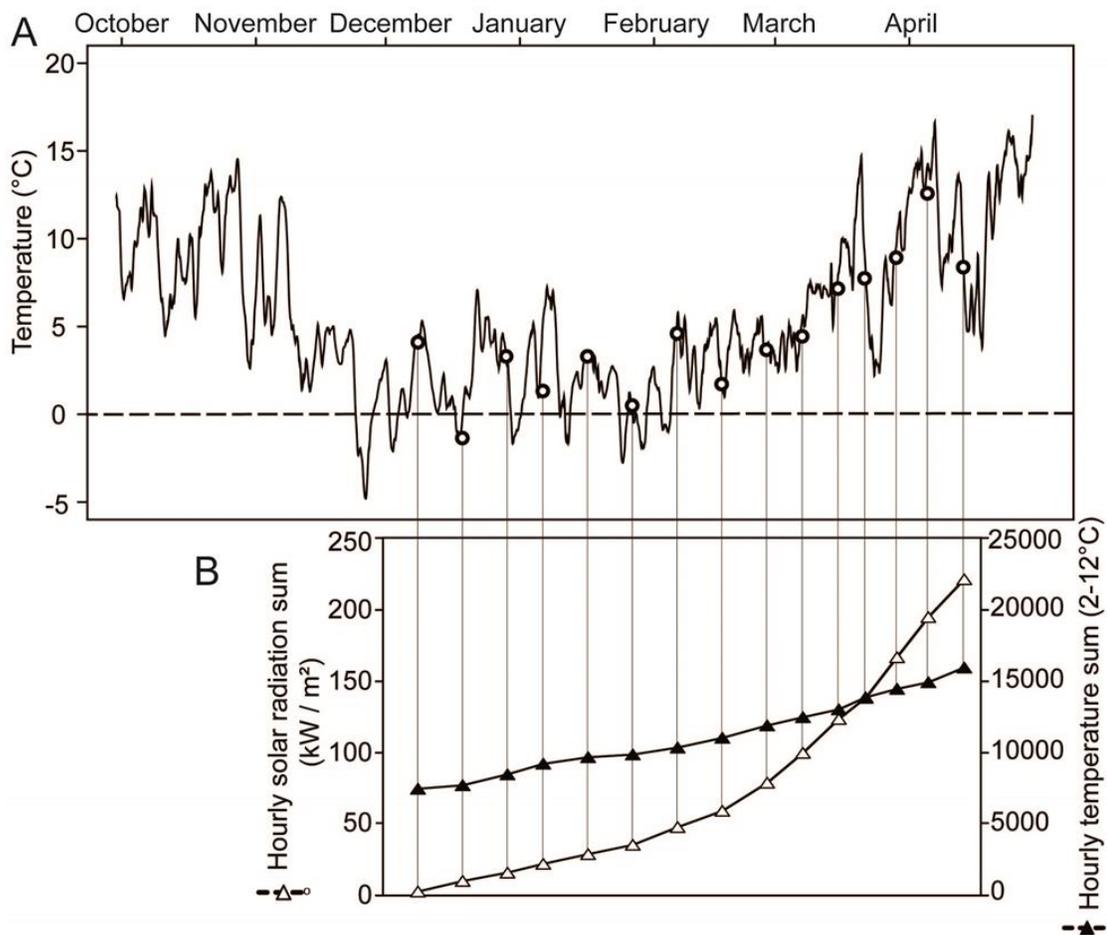
### Overwintering conditions and treatments

Potted seedlings (n= 34 per species and per provenance) were buried in a sand bed to the brims of the pots in the Ecological Botanical Garden of the University of Bayreuth, where they overwintered until spring 2014. Outside ambient temperature at the overwintering site was recorded at plant height (~ 25 cm) with temperature loggers (HOBO Pro v2, Onset Computer Corporation, Massachusetts USA), while global shortwave downwelling radiation ( $W/m^2$ ) was measured at close to the overwintering site at 2 m height at 10 min intervals with an Albedometer (CM14, Kipp & Zonen B.V. Delft - The Netherlands).

### Response parameters

Accumulated hourly temperature sums in the chilling range (between 2 °C and 12 °C) from 1 October and global radiation sums (summed  $W/m^2$  values from 12 December) were calculated for each sampling date (Fig. 2). Temperatures outside the selected range have been shown to be suboptimal at breaking dormancy and thus were not used (Cesaraccio *et al.*, 2004). Budburst at forcing temperatures has been correlated against sampling date (Myking and Heide, 1995; Heide, 1993), degree hour accumulation above a base temperature of 5°C (Wuehlisch *et al.*, 1995) and number of days at or below 5°C (Murray *et al.*, 1989). Yet, in our case, temperature sum yielded the best correlation with days to budburst and was thus used. Frequent sampling without replication was carried out because the pattern of dormancy loss was of

primary importance, rather than the precise quantification of a difference among few points. (Kreyling *et al.*, 2014). Starting on 12 December, every 7-10 days, 2 seedlings from each provenance and species were transferred from the sand bed to climate chambers (Fig. 2). One seedling was kept at a long photoperiod (16 h) and one at a short photoperiod (8 h), both at 16 °C and 20 °C nighttime and daytime temperatures, respectively. It should be noted that more natural light regimes, simulating perpetually increasing day length, could lead to different results, as found for *P. abies*, which was light sensitive under progressively increasing and decreasing photoperiods in another study (Partanen *et al.*, 1998), but not in ours. Light sensitivity can also affect bud burst in a species-specific manner, with *F. sylvatica* again being more sensitive (Caffarra and Donnelly, 2011)



**Figure 2.** Sand bed temperatures at plant height, and temperature sum accumulation (2 to 8 °C) (black triangles) and global solar radiation accumulation (white triangles) up to each sampling date (15 in total), when tree seedlings were transferred from the field site and placed into climate chambers (20 °C) at either short or long photoperiod to determine the number of days to bud burst.

Every 2 days the plants were watered with approx. 50 mL of deionized water per pot and budburst was defined as the first green being visible (budburst stage 3 as described in von Wuehlisch *et al.*, (1995). Midwinter dormancy depth was estimated for every species by calculating the mean days to budburst across first three sampling dates in the middle of winter (23 December, 1 January and 10 January). Occupancy of plants, along with the temperature and photoperiod settings, were alternated every 4 days between the two chambers to minimize potential chamber specific effects. In addition to recording budburst dates inside the chambers, ambient budburst per species and provenance in the field was recorded in the spring.

### Statistical analyses

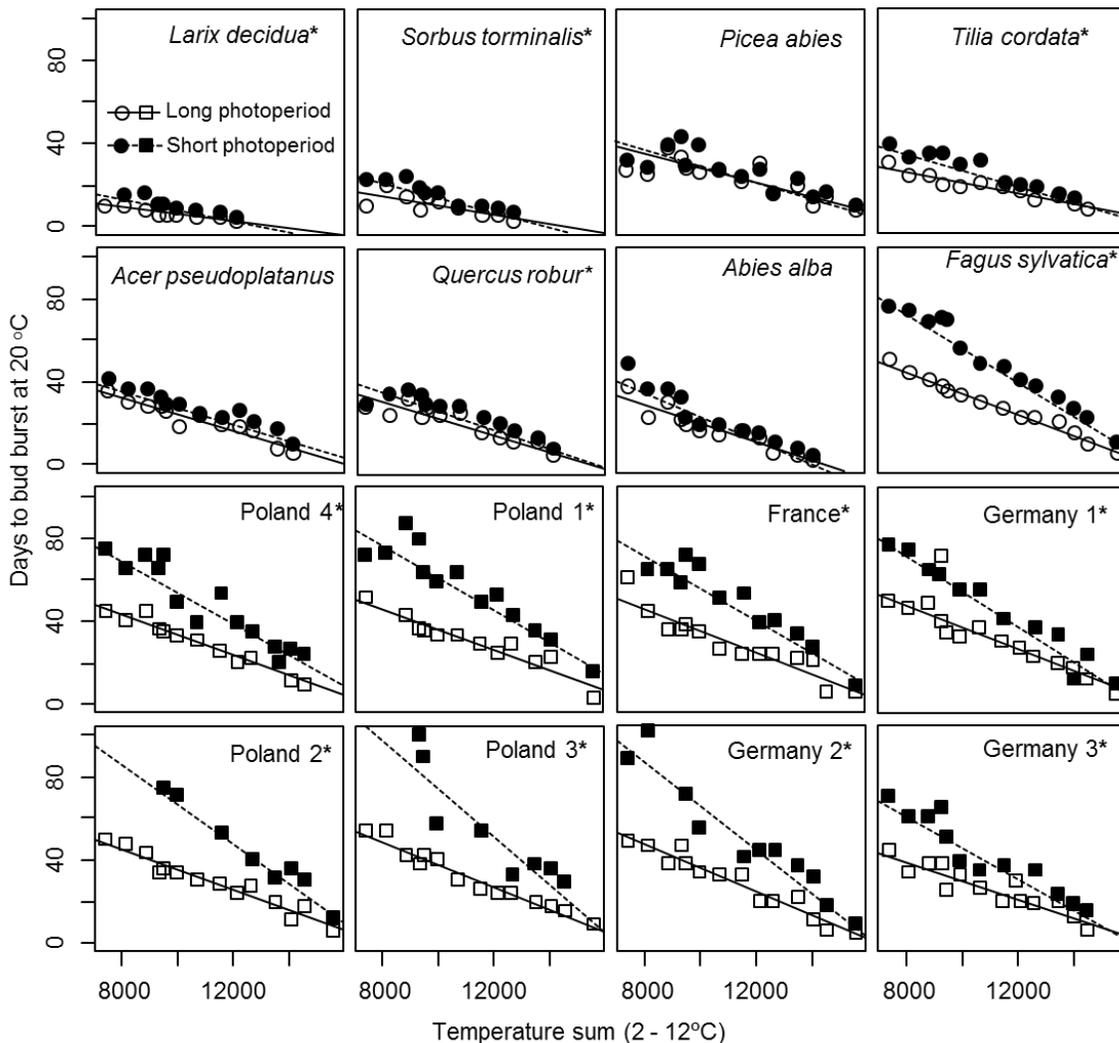
Linear models with temperature sum as the explanatory variable and days to budburst as the response variable were applied for long and short photoperiods separately, for each species and provenance. Correlation coefficients, calculated from linear regressions of temperature sum vs. days to budburst, were subsequently correlated with midwinter dormancy depth (days to budburst at forcing temperature on 10 January). Midwinter dormancy level was likewise correlated with ambient spring budburst dates. Two-way ANOVAs were then run for each species and provenance with days to budburst in climate chambers as the response parameter. Temperature sum at each sampling date and photoperiod length were the fixed factors. Homoscedasticity was checked with residual plots and normality of residuals was tested with normal probability plots (Faraway, 2005). All statistical analyses were performed using R version 3.0.1 (R Development Core Team 2013) and the additional package *sciplot* version 1.1-0.

## Results

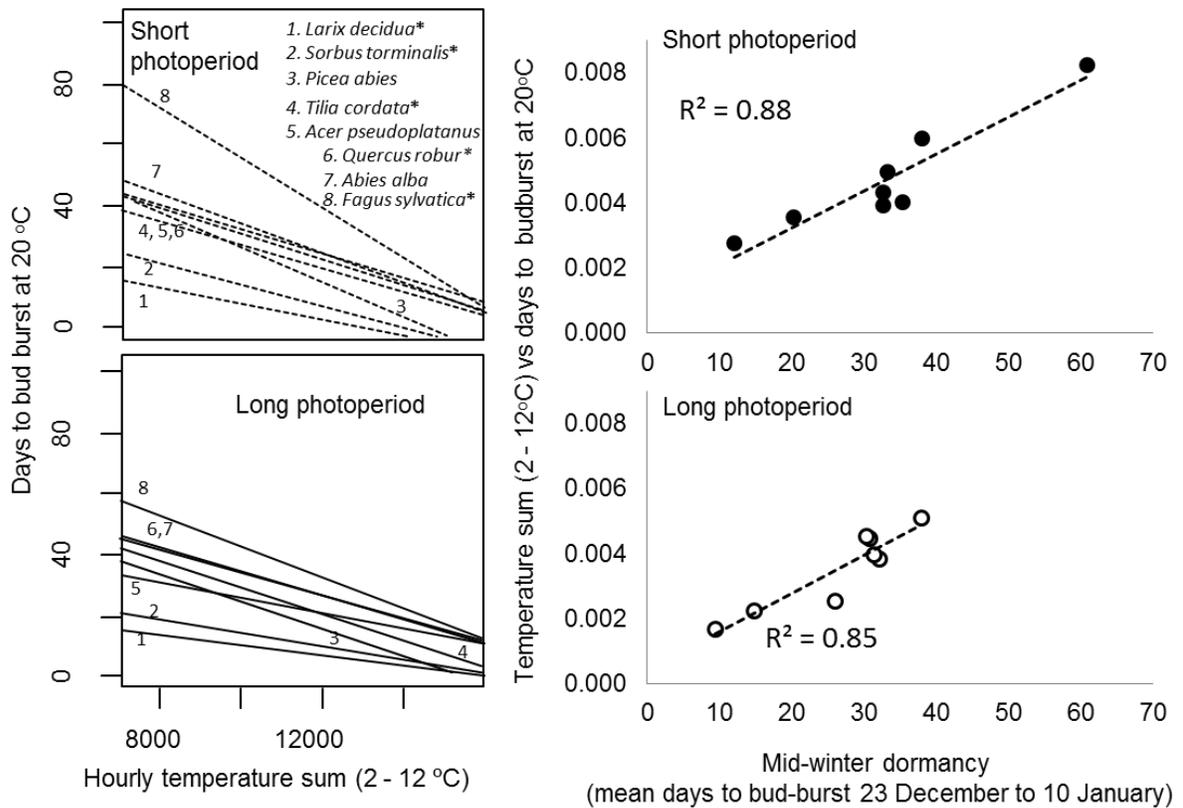
### Species-specific chilling and photoperiod sensitivities

Rates of loss in dormancy depth varied among the 8 species, with less variation within-species, especially at the long photoperiod (Fig. 3 and 4). Likewise, actual budburst dates at ambient field conditions in the spring varied from 14 March to 25 April (Table 3), with much less variation within-species (+ / - 3 days). In six out of eight tree species bud burst was significantly influenced by photoperiod (Fig. 3 and 5; Table 3). The actual mean days to budburst and the corresponding percent decrease were reduced by far the most by the long photoperiod in *F.*

*sylvatica*, followed by *T. cordata* and the rest of the species having relatively little or no sensitivity to photoperiod (Table 3). No differences were found among the provenances of *F. sylvatica* with respect to photoperiod sensitivity. Similar budburst responses to temperature and photoperiod among *Q. robur*, *A. alba*, *P. abies* and *A. pseudoplatanus* allowed us to place the 4 species in the same budburst sensitivity group when ranking the species (Figure 5; Table 3).



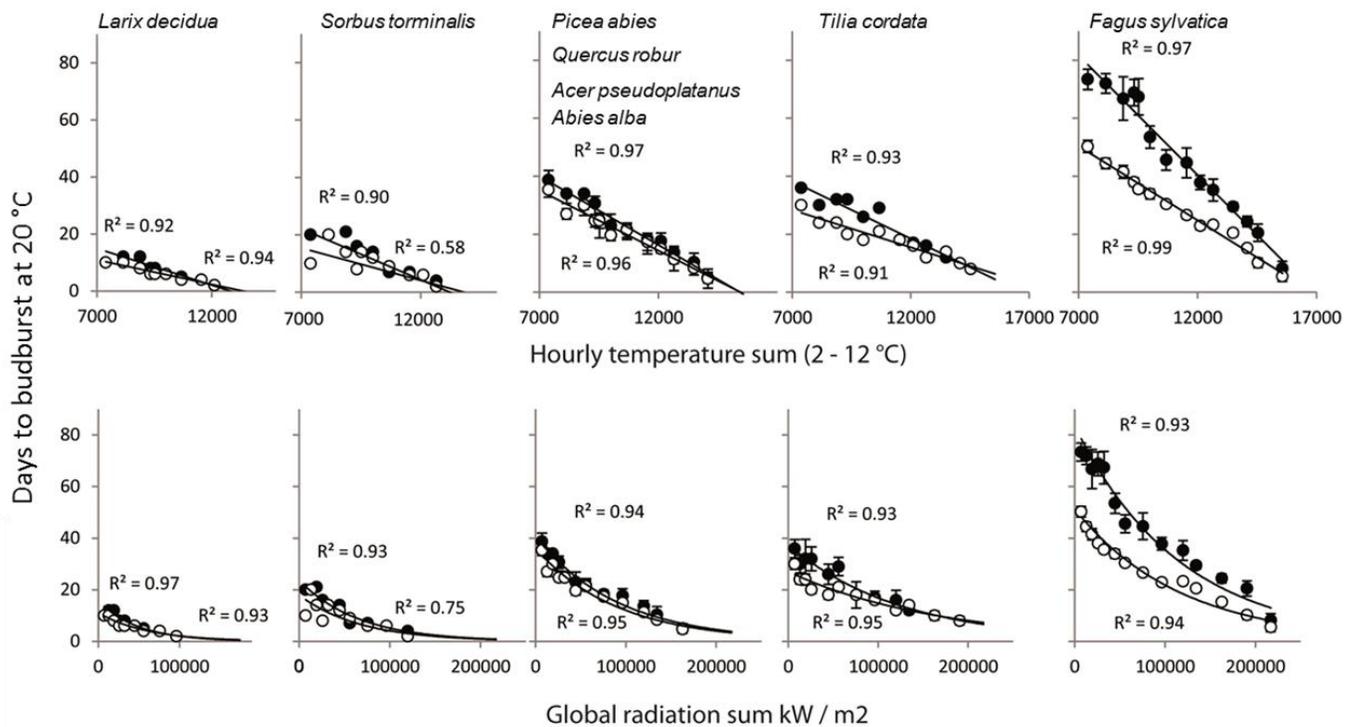
**Figure 3.** Linear regressions between accumulated chilling temperature sums at the overwintering site of tree seedlings and days to budburst in climate chambers at 20°C at weekly sampling dates. Top two rows: among species variation. Bottom two rows: within species variation within *F. sylvatica* (with respective origins). Significantly photoperiod – sensitive species are marked with an asterisk (see Table 3). Geographical and climatic information on each species and provenance is available in Tables 1 and 2, respectively. Y – axis shows days required for tree seedling buds to burst at 20°C in a climate chamber after being brought from the overwintering field site at each of the 14 sampling dates. Species are marked with circles, and provenances of *F. sylvatica* with squares. Days to budburst at short and long photoperiods are marked with black and white circles, respectively.  $R^2$  values were always above 0.8.



**Figure 4.** Relationship between dormancy depth and rate of dormancy loss. Left panel: linear regressions between accumulated temperature sums at the overwintering site of tree seedlings (14 sampling dates) and days to budburst in climate chambers at 20°C, at respective photoperiods. Only the regression lines for each species are shown for clarity. Geographical and climatic information for each species is available in Table 1. Panel B: linear regressions of mid-winter dormancy depth (days required for tree seedlings to burst bud) as a predictor of the rate of dormancy loss (correlation coefficients from Panel A). Each point represents a distinct tree species.

#### Relationship between mid-winter dormancy, rate of dormancy loss and spring budburst date

At both photoperiods and across species, the dormancy depth mid-winter (mean days to budburst from December 2 to January 10) was positively correlated with rate of dormancy loss (rate of reduction in number of forcing days required to bud burst with accumulated chilling sum; Fig. 4), as evident from the species correlation coefficients from the linear regressions of chilling temperature sum vs. days to bud burst. The same correlation across provenances of *F. sylvatica* was not significant. Mid-winter dormancy depth was also positively correlated with spring budburst dates across species (at short photoperiod:  $p = 0.086$ ,  $\text{adj } R^2 = 0.32$ ; at long photoperiod:  $p = 0.008$  and  $\text{adj } R^2 = 0.49$ ), with a weaker correlation among provenances of *F. sylvatica* only being significant at the long photoperiod ( $p = 0.017$ ,  $\text{adj. } R^2 = 0.11$ ) (Table 3).



**Figure 5.** Budburst responses of species grouped according to the 5 patterns observed, based on differences in midwinter dormancy depth, rate of dormancy loss and photoperiod sensitivity. Pooled budburst responses for tree seedlings across species (*P. abies*, *Q. robur*, *A. pseudoplatanus* and *A. alba*) and across *F. sylvatica* provenances are shown due to similar responses among these species and provenances, respectively. Days to bud-burst at short and long photoperiods are marked with black and white circles, respectively. Linear regression coefficients are displayed for correlations with temperature sum increases, while exponential correlation coefficients are presented for correlations with solar sum increase. Not all sampling points are displayed for all species, because specific species started bursting outside naturally (see Table 3 for ambient budburst dates). Error bars indicate standard error.

### Photoperiod sensitivity changes from winter to spring

The effect of photoperiod decreased linearly with accumulated chilling sum and exponentially when plotted against radiation sum (Fig. 5). Statistically, the effect of photoperiod decreased with accumulated chilling temperature sum in 2 of the 5 temperature sensitive species (Table 3).

**Table 3:** Summary of species-specific photoperiod sensitivities (significant differences between short and long photoperiods) with respective effect sizes (days by which budburst was delayed under short photoperiod and the corresponding relative percent time delay). Dependence of the photoperiod sensitivity on the chilling requirement is represented by significant interactions between photoperiod sensitivities and chilling temperature sum. Relative natural budburst dates: days since March 1 when each species burst bud outside at ambient conditions (n= 4). Midwinter dormancy is represented by mean days to budburst (mean of short and long photoperiod days) from December 23 to 10 January. Significant differences and interactions are marked in bold ( $p \leq 0.05$ ). Shading intensity represents possible relative budburst sensitivities to warmer winter and spring (decreasing sensitivity with darker shading), based on both midwinter dormancy depth, rate of dormancy loss and photoperiod sensitivity.

Species	Photoperiod sensitive?	Photoperiod sensitivity depends on chilling requirement?	Decrease in mean days to budburst at long photoperiod	Percent decrease in mean days to budburst at long photoperiod	Mean days to budburst at forcing temperature mid-winter (short / long photoperiod)	Relative natural budburst date at ambient conditions
<i>Larix decidua</i>	<b>Yes</b>	<b>No</b>	<b>1.6</b>	<b>22%</b>	9/12	13
<i>Sorbus torminalis</i>	<b>Yes</b>	<b>No</b>	<b>2.8</b>	<b>22%</b>	15/20	23
<i>Quercus robur</i>	<b>Yes</b>	<b>No</b>	<b>2.3</b>	<b>(10%)</b>	30/33	44
<i>Picea abies</i>	No	No	0	(1%)	32/33	55
<i>Acer pseudoplatanus</i>	<b>Yes</b>	<b>No</b>	<b>3</b>	<b>(12%)</b>	31/35	45
<i>Abies alba</i>	No	No	2.8	14%	30/38	35
<i>Tilia cordata</i>	<b>Yes</b>	<b>Yes</b>	<b>5.5</b>	<b>24%</b>	26/33	56
<i>Fagus sylvatica</i> (provenance mean)	<b>Yes</b>	<b>Yes</b>	<b>17.8 (+/-1)</b>	<b>39% (+/- 2)</b>	38/61	56 (+/-1)

## Discussion

Depth and loss rate of winter dormancy are correlated

*P. abies*, *T. cordata* and *F. sylvatica* had very different mid-winter dormancy depths, especially at the short photoperiod, yet all burst on almost the same day in the spring at ambient

conditions (Table 3). This happened due to the positive correlation between midwinter dormancy depth and rate of loss in dormancy depth from winter to spring, a pattern present in all species and provenances of *F. sylvatica*. This correlation meant that our first hypothesis was supported and even at the sub-species level, seedlings genetically programmed to achieve higher mid-winter dormancy also lose it faster as an adaptation to minimize budburst delay in the spring. Without such an adaptation, the growth delay would be much greater (Panels A and B in Fig. 1). Indirect support for this relationship is provided by Laube *et al.* (2014), where the ranking of species according to budburst sensitivities to forcing temperatures changed from early to late winter. Earlier studies have also shown similar patterns but no discussion was made regarding the possible generality as an adaptation (Heide, 1993a; Murray *et al.*, 1989) However, this apparent adaptation does not completely compensate for having deeper mid-winter dormancy, because a positive relationship was still found between dormancy depth and later spring budburst date, albeit only under the long photoperiod conditions.

#### Linear slopes of decrease in dormancy depth

The linear relationship between the depth of tree bud dormancy and accumulated chilling sums from mid-winter to spring show the gradual nature of dormancy depth decrease with increased accumulation of chilling temperature sums (Myking, 1999; Myking and Heide, 1995). Viewing and quantifying dormancy as a dynamic process, rather than its presence or absence (Thomas and Vince-Prue, 1996), is therefore important in understanding bud phenology. Previous studies have shown that the decrease in forcing requirements occurs much faster in early winter than at the onset of spring, following an exponential pattern (Murray *et al.*, 1989; Myking and Heide, 1995b; Vitasse and Basler, 2013). A linear pattern has been observed for *F. sylvatica* however (Vitasse and Basler, 2013), and non-linear patterns for other species were only apparent after accumulation of 40 chilling days or less (Myking and Heide, 1995; Vitasse and Basler, 2013). In our experiment, due to the mild winter, sampling commenced after already more than 70 chilling days had accumulated. Sampling had therefore largely taken place within the right-tailed portion of the potentially exponential curves. In any case, between-species comparisons and ranking should not be affected by this discrepancy.

### Photoperiod sensitivity is stronger in winter than in spring

Our results confirmed our second prediction, in that photoperiod sensitivity decreased linearly with accumulated chilling temperatures and exponentially when plotted against the solar radiation sum. Also, as we predicted, *F. sylvatica*'s highest photoperiod sensitivity exemplified this trend, agreeing with results of decreased photoperiod effect with increasing accumulation of chilling temperatures. These findings highlight the decrease in photoperiod sensitivity from mid-winter to spring, supplementing general previous findings of lower photoperiod influence on budburst in the spring (Caffarra and Donnelly, 2011; Heide, 1993a). As a consequence, many tree species (with perhaps the exception of *F. sylvatica* and to a lesser extent *T. cordata*) will continue to respond to warmer spring temperatures with similar rates of advancing budburst, until budburst starts as early as the period when photoperiod sensitivity still plays a role. This result also agrees with previous reports of high photoperiod sensitivity at low chilling accumulation stages (i.e. reduction of forcing requirements with photoperiod only at the low chilling accumulation stage - (Laube *et al.*, 2014; Vitasse and Basler, 2013), which additionally highlights the linear trend.

### Photoperiod sensitivity is positively correlated with mid-winter dormancy depth

We predicted that high midwinter dormancy and high photosensitivity would be positively related, because other tree species have also shown the tendency for high photoperiod sensitivity in combination with high chilling requirements (Laube *et al.*, 2014). Our results do not support this trend, showing that species with low midwinter dormancy depth may still have photoperiod sensitivity similar to species with much higher midwinter dormancy (scenario D does not always hold true in Fig. 3). This conclusion has to be interpreted with caution, however, due to our small sample size of photoperiod sensitive species (6). Furthermore, only 2 species had photoperiod sensitivity with an appreciable effect size (a mean of greater than 3 days for budburst with respect to photoperiod). Therefore, whether the effect of photoperiod is directly proportional to the dormancy depth cannot be answered with our data. Testing more photoperiod-sensitive species is

required to determine if the dormancy depth / chilling requirements may also largely determine the level of photoperiod sensitivity.

#### Among-species differences

The well-known high chilling requirements of *F.sylvatica* (Dantec *et al.*, 2014; Fu *et al.*, 2012; Heide, 1993b) were confirmed in our study, with the additional finding that this species also possesses the highest rate of dormancy loss (at least among the species in this study).

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The six species found to be photoperiod sensitive have largely been described as such in the literature (Way & Montgomery, 2014 and the references within), although other *Sorbus* species and *L. decidua* have been previously found to not be photoperiod-sensitive (Heide, 2011; Basler and Koerner, 2012), while *A. alba* has been shown to be photoperiod sensitive (Laube *et al.*, 2014). This discrepancy could lie in the specific species of *Sorbus* studied, and the methodology used. Heide (2011) focused on the rate of growth under different photoperiod conditions, while Basler and Koerner (2012) used chilled twigs instead of potted seedlings. In any case, the effect size of photoperiod was minimal in both species (mean differences of 1.6 and 2.8 days to budburst). *A. alba* is also likely to be photoperiod-sensitive, but high variation in budburst dates resulted only in a marginally significant photoperiod effect ( $p = 0.08$ ).

#### Within-species differences

*F. sylvatica* provenances had the same correlation patterns between midwinter dormancy and rate of dormancy loss as observed among-species. Light sensitivity and mid-winter dormancy levels were also similar, suggesting strong genetic control for chilling requirements and photoperiod sensitivity (Chmura and Rozkowski, 2002; Gömöry and Paule, 2011). Previous studies have shown a longitudinal cline exists for spring bud burst in *F. sylvatica*, with the earliest budburst occurring in eastern provenances (Gömöry and Paule, 2011; Wuehlisch *et al.*, 1995). There might be stronger differentiation among populations and provenances in more

southern beech distribution range where genetic variation in *F. sylvatica* is much stronger than in Central European range. This can be explained due to the idea that central Europe was colonized after the last ice age from only small populations in one refuge with a limited number of genotypes (Magri *et al.*, 2006).

#### Implications for climate change responses

*F. sylvatica*, which is the naturally dominant tree species in central Europe (Barr *et al.*, 2004; Rollinson and Kaye, 2012). Our results show that this may be true for most of our studied species, because the chilling hour accumulation had a much greater effect in reducing the dormancy level, compared with the photoperiod change. For *F. sylvatica*, however, the influence of photoperiod was much greater than for any other species and may cause comparatively stronger limits of phenological advances with warmer temperatures. As a consequence, *F. sylvatica* will potentially be at a disadvantage relative to other much less photoperiod-sensitive species. Adult trees typically flush later than seedlings which we examined (Vitasse, 2013) and although the species budburst rankings will likely be similar, the effect sizes will likely change.

The photoperiod sensitivity of *F. sylvatica* also extended the longest, until April, whereas the other species were photoperiod insensitive by March. *F. sylvatica* is therefore likely to be the least sensitive species - among those tested - to extreme warming events in mid-winter and spring, which can lead to the onset of premature growth and higher susceptibility to frost events. Warming events in winter may lead to loss of frost hardiness (Hughes *et al.*, 2008) and more frost damage afterwards (Bokhorst *et al.*, 2009; Schuerings *et al.*, 2014). *F. sylvatica* will likely not be susceptible to mid-winter sudden warm spells due to high dormancy depth at this time. With respect to frost events in spring, it is projected that the intensity and duration within this century will be the same as in the last century (Kodra *et al.*, 2011). In addition, despite a decreasing frequency of frost events, the frequency and intensity of frost damage is increasing (Augsburger, 2013; Gu *et al.*, 2008; Inouye, 2008).

On the other hand, *F. sylvatica* may also advance its spring phenology at a slower rate than other tree species, as previously reported (Aber *et al.*, 2001; Heide, 1993b; Vitasse, 2013), which

would be a disadvantage in the absence of frost. The low budburst plasticity of *F. sylvatica* to changes in temperatures preceding budburst (Vitasse and Basler, 2013) is likely to be driven by both higher chilling requirements and higher photoperiod sensitivity, as supported by our results. It is clear, however, that such species are likely to exhibit the lowest phenological sensitivities to warming, and are also likely to be more sensitive to budburst delays, which have been shown to occur when warmer winter temperatures are insufficient to meet the chilling requirements of such species (Yu *et al.*, 2010).

We were able to assign our 8 species to 5 categories, according to how sensitive the species might be to an advancing spring and midwinter warming. Increasing midwinter dormancy depth and photoperiod sensitivity were assumed to limit the ability of a species to track warmer temperatures. *L. decidua* can therefore be expected to be followed by *S. torminalis* as the second most warming-sensitive species, due to higher midwinter dormancy and photoperiod sensitivity (Table 3). Next follow the four late successional species, *Q. robur*, *P. abies*, *A. pseudoplatanus*, and *A. alba*; *P. abies* may be the leastwarming sensitive among these four species due to no detected photoperiod effect. *T. coradata* had very similar midwinter dormancy to these species, but was considerably more photoperiod sensitive and was therefore the second most warming-sensitive species. *F. sylvatica* had by far the deepest midwinter dormancy and highest photoperiod sensitivity, potentially making this species the least warming sensitive among the common European tree species. Short-lived, pioneer species generally have low chilling requirements for budburst and high sensitivity to forcing temperatures, bursting rapidly at equal forcing conditions (Caffarra and Donnelly, 2011, Laube *et al.*, 2014). Such species may become more dominant at the expense of more photoperiod sensitive species, and species with higher chilling requirements (Morin *et al.*, 2009). *L. decidua* has been suggested to have an advantage over less photoperiod-sensitive species (typically late successional species) by being able to take advantage of warmer winters (Koerner and Basler, 2010; Laube *et al.*, 2014), which was also supported by our results.

## Conclusions

We observed a general trend among species with respect to dormancy depth and rate of dormancy loss. Species achieving higher mid-winter dormancy may be adapted to also lose their

bud dormancy faster with the onset of the growing season, thereby minimizing any growth delay in the spring. Furthermore, four late succession species had very similar dormancy loss patterns and photoperiod sensitivities, likely causing them to react similarly to warmer winter and spring. With respect to climate warming implications, we presented a species ranking, with *L. decidua* likely to exhibit the strongest positive growth response to advancing spring and warmer winters. *F. sylvatica* would likely be the most insensitive to mid-winter warming, due to its low warming sensitivity and high photoperiodic sensitivity at this time. In the spring, the responses of the studied species to warming would likely be more similar, due to 1) the faster dormancy loss rates of the more dormant species and 2) the diminishing influence of photoperiod sensitivity across species

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## **Manuscript 2: Relative effects of temperature vs. photoperiod on growth and cold acclimation of northern and southern ecotypes of the grass *Arrhenatherum elatius***

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### Highlights:

- Temperature is more important than photoperiod for cold acclimation of a grass.
- Northern ecotypes are more responsive to cold acclimation cues.
- Ecotypic cold acclimation differences in *A. elatius* resemble tree patterns.
- For biomass production, faster growth pre-winter outweighs frost damage effects.

### Abbreviations

Temp<sub>low</sub> – low acclimation temperature (3 °C)

Temp<sub>high</sub> – high acclimation temperature (8 °C)

Temp<sub>high+low</sub> – high acclimation temperature (8 °C),  
followed by low acclimation temperature (3 °C)

Photo<sub>short</sub> – short acclimation photoperiod (6 hours)

Photo<sub>long</sub> – long acclimation photoperiod (12 hours)

### Abstract

Growth of perennial grasses in the fall represents a balance between an extended growing season and increased vulnerability to frost. Within species along latitudinal gradients, plants may exhibit ecotype-specific sensitivities to the temperature and photoperiod cues that influence cold acclimation. Therefore, it is unclear for a given latitude how climate warming will alter the timing and extent of cold acclimation, and thus vulnerability to frost events. We evaluated

relative temperature and photoperiod sensitivities during simulated cold acclimation for two northern (Swedish) and two southern (Italian) ecotypes of the common forage grass *Arrhenatherum elatius*. Three temperature levels (Temp<sub>low</sub>: 3 °C, Temp<sub>high+low</sub>: 8 °C followed by 3 °C, and Temp<sub>high</sub>: 8 °C) were crossed with 2 photoperiod levels (Photo<sub>short</sub>: 6 h, and Photo<sub>long</sub>: 12 h) and administered to the plants for a three week acclimation period. All plants were then frozen at -8 °C for 1 day, and post-frost growth was measured after 3 weeks. Temp<sub>high</sub> and Photo<sub>long</sub> increased growth prior to frost, but resulted in decreased growth after frost. The effects of temperature on sugar concentration, biomass and flower presence depended on photoperiod, with temperature only influencing sugar concentration and flowering at Photo<sub>short</sub>, while Photo<sub>long</sub> increased biomass only at the high temperature. The faster growth rate of southern *A. elatius* ecotypes before frost, in combination with sufficient cold acclimation, resulted in higher biomass accumulation after frost. The faster growth habit of southern ecotypes may be advantageous in accumulating higher summer biomass even after moderate frost events in the fall.

**Keywords:** cold acclimation, perennial grasses, intra-specific variation, latitudinal ecotypes, photoperiod, temperature.

## 1. Introduction

Global mean temperature is predicted to increase by 1.1 to 6.4 °C by 2090-2099 relative to 1980-1999 (IPCC, 2007), contributing to an extended plant growing season, in part through delays in fall senescence (Smithberg and Weiser, 1968; Jeong et al., 2011; Vitasse et al., 2011). In the northern hemisphere, delayed fall senescence and accelerated spring growth was documented between 1982 and 2008 (Jeong et al., 2011). However, different deciduous tree species have shown variable sensitivities to changes in temperature and photoperiod (Vitasse et al., 2009); for example, among shrub and liana species common to deciduous forests in the eastern United States, non-native species have extended their growing seasons longer into the fall than some native species (Fridley, 2012). In addition, differences in growth requirements are responsible for variation in the delay of leaf senescence in trees (Juknys et al., 2012). Overall, the definitive determinants of plant senescence, and in particular their interactions, are unclear for many species (Estrella and Menzel, 2006), and thus warrant further study.

Reductions in plant growth in the fall are closely linked with increasing plant cold acclimation (Weiser, 1970). When and how quickly plants acclimate to cold both have an impact on plant vulnerability to frost, as well as on the maximum level of cold tolerance that can be achieved. (Kalcsits et al., 2009). Warmer fall temperatures can delay and decrease cold acclimation, increasing the vulnerability of plants to frost damage (Linden, 2001). In addition to temperature, photoperiod is an important cue for cold acclimation (McKenzie et al., 1988; Stout and Hall, 1989). Some woody perennials are able to cold acclimate under declining photoperiod alone (Palonen, 2006), but others are primarily driven by temperature (Kaurin et al., 1982), and short photoperiod enhances the effect of low temperature in some tree species (Welling et al., 2002). The relative influences of photoperiod vs. temperature on plant cold acclimation could determine the extent to which climate warming in the fall will likely alter the timing and rate of cold acclimation, but unlike warming, photoperiod cycles at a given location will remain constant. Despite substantial interspecific variation in the roles of photoperiod vs. temperature on plant acclimation, several trends have emerged, such as the dominance of photoperiod in initiating plant senescence (Lagercrantz 2009), although these trends have been based almost entirely on woody plant acclimation responses. Photoperiod is also typically more influential than temperature as a growth cue in late vs. early successional tree species (Basler and Koerner, 2012), and in northern vs. southern species (Junttila, 1982; Howe et al., 1995). However, plant cold acclimation responses may differ between herbaceous and woody plants (Welling et al., 2002).

Intra-specific variation in responses to cold acclimation cues also exist (Kalcsits et al., 2009), particularly among ecotypes (i.e. distinct populations that are adapted to a local environment (Hufford and Mazer, 2003). For example, tree ecotypes from northern latitudes can acclimate faster than southern ecotypes (Li et al., 2005), and are more sensitive to temperature changes (Howe et al., 2000; Junttila et al., 2003). However, very few studies have focused on the relative influences of photoperiod and temperature on the cold acclimation of grass ecotypes from different latitudes. Differences in cold acclimation among grass ecotypes are important to examine, given that perennial temperate grass species cover a broad geographic range, grow under variable conditions and include climatically-adapted ecotypes (Macel et al., 2007; Ofir and Kigel, 2010; Beierkuhnlein et al., 2011). Spring frost tolerance, for instance, was less developed

in individuals originating from sites with warmer spring temperatures than in individuals originating from colder spring temperatures in two out of four temperate grass species (Kreyling et al., 2012).

We examined the relative temperature and photoperiod sensitivities of northern (Swedish) and southern (Italian) ecotypes of the common forage grass, *Arrhenatherum elatius*, during the fall cold acclimation period. *A. elatius* is wide-spread across Europe and is important for agriculture as a key forage species in permanent grasslands (Beierkuhnlein et al., 2011). We focused on plant performance both before and after a simulated fall frost event, and compared the growth responses to the different cold acclimation regimes. Regarding treatment effects, we hypothesized that long photoperiod and the warmest temperature treatment would lead to the greatest growth prior to frost and the lowest growth after the frost treatment, due to greater frost damage. We further hypothesized that northern ecotypes would acclimate faster than southern ecotypes, leading to decreased growth before the frost event, but increased relative growth after the frost event.

## **2. Methods**

### 2.1 Plant cultivation

Two northern (from Sweden) and two southern (from Italy) ecotypes of *A. elatius* (Table 1) were cultivated from January to February 2013 at the Leibniz Institute of Plant Genetics and Crop Plant Research in Poel, Germany. Plants were germinated over a two week period starting the end of December on filter paper in a climate chamber set at close to 100% humidity, with a 12 h photoperiod and mean day and night time temperatures of 22 °C and 15 °C, respectively. Seedlings were then transplanted into seed compost soil on 3 January (Classic Profisubstrate, Einheitserde, Germany) in plastic pots (5 cm diameter × 7 cm deep). NPK (Mg) liquid fertilizer (15+10+15+ (2)) was applied once at a concentration of 1 g/L (Hakaphos Blau, Compo expert, Germany). Until the end of February the plants were grown in a greenhouse where day and night temperatures averaged 19.6 °C +/- 0.9 °C and 9.6 °C +/- 0.9 °C (standard error), respectively. Ten hour photoperiod was provided with 400 W lamps, with plants being trimmed twice. Plants were transferred to climate chambers in Bayreuth, Germany at the end of February for the start of

the acclimation treatments, with a light intensity of 180  $\mu\text{mol}/\text{m}^2/\text{s}$ . Plants were trimmed to a height of 2 cm prior to starting the acclimation treatments.

**Table 1.** Environmental variables of the origins of ecotypes for *A. elatius* used in the experiment. Climate data for 1950-2000 from worldclim (<http://www.worldclim.org/>). MAT: Mean Annual Temperature. Annual temperature range is the difference between the mean yearly maximum and minimum temperatures. MTWQ: Mean Temperature of Warmest Quarter. MTCQ: Mean Temperature of Coldest Quarter.

Location	Latitude	Longitude	Elevation (m)	MAT (°C)	MTWQ (°C)	MTCQ (°C)	Mean minimum temperature of coldest month (°C)	Annual temperature range (°C)
<b>Kramfors, Sweden</b>	17.7921	62.9361	20	3.0	14.1	-7.9	-13.0	33.0
<b>Alnön, Sweden</b>	17.4103	62.4469	102	3.3	14.2	-7.7	-12.9	33.2
<b>Colfiorito, Italy</b>	12.8782	43.028	762	10.6	19.0	2.7	-0.4	25.3
<b>Rusino, Italy</b>	10.2583	44.5028	1100	10.9	19.7	1.9	-1.8	27.8

## 2.2 Plant acclimation treatments

The experiment had 3 temperature treatments  $\times$  2 photoperiod treatments  $\times$  2 latitudes in a factorial design. A 6 h photoperiod difference was chosen to represent the maximum yearly photoperiod difference for the southern ecotypes and the photoperiod difference from September to November for the northern ecotypes, while optimal and suboptimal acclimating temperatures were used for the temperature levels (Paquin and Pelletier, 1980). Ecotypes were cold acclimated for 3 weeks at 6 different regimes: (1) long (12 h) photoperiod and high (8°C) temperature (Photo<sub>long</sub> ; Temp<sub>high</sub>), (2) long (12 h) photoperiod and low (3°C) temperature (Photo<sub>long</sub> ; Temp<sub>low</sub>), (3) short (6 h) photoperiod and high (8°C) temperature (Photo<sub>short</sub> ; Temp<sub>high</sub>), (4) short (6 h) photoperiod and low (3°C) temperature (Photo<sub>short</sub> ; Temp<sub>low</sub>), (5) 1.5 weeks of regime 1 followed by 1.5 weeks of regime 2 (Photo<sub>long</sub> ; Temp<sub>high+low</sub>), (6) 1.5 weeks of regime 3 followed by 1.5 weeks of regime 4 (Photo<sub>short</sub> ; Temp<sub>high+low</sub>) (n=10 per treatment, per ecotype). Acclimation regimes 1 to 4 were administered in individual climate chambers. For a given regime, the plants were transferred to a different chamber every 5 d (with the growth conditions

changed accordingly) to distribute the potential effects of chamber differences among treatments. The measured mean temperatures of the above acclimation regimes, incorporating climate chamber temperature changes, were as follows: (1) 8.4 °C, (2) 3.6 °C, (3) 8.6 °C, (4) 3.2 °C, (5) 5.3 °C and (6) 5.3 °C.

### 2.3. Frost event and post frost growth conditions.

At the conclusion of the cold acclimation treatments, a freezing event (freezing and thawing rates of 1 °C/h, starting and ending at 4 °C, with a minimum temperature of -8 °C held for 8 h) was administered to all plants in one climate chamber. Plants were repotted into larger pots (8 cm × 8 cm × 20 cm) using the same soil and transferred to a greenhouse where temperature averaged 8.5 °C +/- 0.2°C (standard error) for the first 10 d and 17.0°C +/- 0.2°C for the next 27 d (min and max temperatures were 7.0 °C and 32 °C respectively). Taken together, the experiment artificially comprised a shortened full annual cycle (spring germination, summer growth, fall acclimation, winter frost, spring onset of growth, summer flowering).

### 2.3 Response parameters

Growth performance following frost exposure has previously been used as a relative measure to evaluate the effectiveness of plant cold acclimation (Malyshev and Henry, 2012). Plant height was measured prior to the frost at the end of the acclimation treatments, and again one month after the frost treatments. The measurements taken at the end of the acclimation period and before the frost were used to quantify growth differences during acclimation. The average heights of 3 leaves per plant were recorded. Relative plant height change was calculated by subtracting the final height after the frost from the height prior to frost and dividing the difference by the height prior to frost. This measure was used to evaluate the post-frost growth independent from the growth during the acclimation period. Aboveground biomass was harvested 7 d after the post-frost height measurement (5 weeks after the frost event). Biomass therefore comprised the full effect of the response to both acclimation and frost. Plant material was dried at 60 °C to a constant biomass and weighed. Flower presence was noted for each plant immediately prior to the biomass harvest.

Total leaf soluble sugar concentration was determined just prior to the frost as a possible indicator of cold acclimation status (Sauter et al., 1996; Gusta et al., 2004). Three leaf blades approximately 5 cm in length were cut and dried at 60 °C to a constant weight. Sugar content was assessed using the phenol-sulphuric acid method (DuBois et al., 1956) modified by Buysse and Merckx (1993). A standard curve was produced using known sucrose concentrations between 0 µg to 200 µg ml<sup>-1</sup>, at 25 µg increments. Approximately 5 mg of dried *A. elatius* leaves were ground (Retsch MM2 Pulverizer Mixer Mill, Retsch GmbH, Germany) for 7 minutes (in Eppendorf tubes) at a rate of 75 strokes per second. Soluble sugars were extracted by taking 5 mg subsamples and incubating them in 1.5mL 80 % ethanol solution overnight. The solution was centrifuged at 5000 × g for 10 minutes. The supernatant was removed, and then re-centrifuged in another 1.5mL 80 % ethanol. The supernatants were combined and kept cool in a freezer for 1 week in tightly closed snap cap vials (15 ml, 52 × 24 mm, 22 mm snap cap, VWR International). Half of one mL of supernatant was transferred to a glass tube, along with 0.5 mL 80% ethanol (dilution was required to obtain the proper range of concentrations for the analysis). One milliliter of 28 % phenol solution was then added, and 5 mL of concentrated sulfuric acid was added immediately after, directing the stream on the surface of the liquid. The solution was shaken using a vortex mixer and allowed to stand for 15 minutes before measuring absorbance at 490 nm.

#### Relative temperature and photoperiod sensitivity analysis

To evaluate the ecotypic differences in temperature sensitivities (pooled by latitude) for different cold acclimation treatments, the ratios relating individual plant responses to mean plant response at colder temperatures were calculated (generating a response value for each plant for statistical analysis); Temp<sub>high</sub> to Temp<sub>low</sub> and Temp<sub>high+low</sub> to Temp<sub>low</sub>, were calculated separately within-Photo<sub>long</sub> and within-Photo<sub>short</sub>. The ratios were calculated for each response parameter (pre-frost height, sugar accumulation, post-frost relative growth and biomass). To evaluate the ecotypic differences in photoperiod sensitivities, the ratios relating individual plant responses to mean plant responses at the shorter photoperiod were calculated; Photo<sub>long</sub> to Photo<sub>short</sub> ratios were calculated separately within each temperature level.

## Statistical analyses

ANOVAs based on linear mixed effects models were used to test cold acclimation treatment effects on plant latitude with respect to plant height before frost, relative change in plant height, total soluble sugar concentration and aboveground biomass. Photoperiod ( $\text{Photo}_{\text{long}}$  and  $\text{Photo}_{\text{short}}$ ), temperature ( $\text{Temp}_{\text{low}}$ ,  $\text{Temp}_{\text{high+low}}$  and  $\text{Temp}_{\text{high}}$ ) and latitude (Italian and Swedish origins) comprised the fixed factors, while the ecotypes and mother groups (plants sharing the same mother) were inserted as random factors. Flower presence/absence was analyzed by binomial generalized linear mixed models. Random and fixed effects were set the same way as for the linear mixed models above. Homoscedasticity of residuals was checked with residual plots and normality of residuals was tested with qq-plots (Faraway, 2005). Data were square root transformed for the biomass and rank transformed for the sugar concentration parameter to satisfy the normality assumption. Repeating the analyses for all response parameters without pooling ecotypes into latitudes produced largely the same response patterns, with temperature and photoperiod having a greater influence on plant response than ecotype, and the only significant interaction between ecotype and temperature existing for biomass (data not shown). All statistical analyses were performed using R (version 2.12.2) and the additional packages *sciplot* (R package version 1.1-0), *nlme* (R package version 3.1-111) and *lme4* (R package version 0.999999-2).

## **3. Results**

### 3.1 Overall cold acclimation effects

$\text{Temp}_{\text{high}}$  and  $\text{Photo}_{\text{long}}$  treatments both independently increased plant height before frost (Table 2 – height before frost; Figure 1A). The 6 h difference in photoperiod increased height by 8 %, while mean temperature increases of 2 °C ( $\text{Temp}_{\text{high+low}}$  vs.  $\text{Temp}_{\text{low}}$ ) and 5 °C ( $\text{Temp}_{\text{high}}$  vs.  $\text{Temp}_{\text{low}}$ ) increased height by 22 % and 37%, respectively. The reverse trend was observed after the frost event for relative change in height, with higher temperature and longer photoperiod treatments during the acclimation period leading to smaller height increases after the frost event (Figure 1 B);  $\text{Photo}_{\text{short}}$  plants grew 10% more after frost than  $\text{Photo}_{\text{long}}$  plants, while  $\text{Temp}_{\text{low}}$  plants grew 15% and 56% more than  $\text{Temp}_{\text{high+low}}$  and  $\text{Temp}_{\text{high}}$  plants, respectively (Table 2 – relative height change; Figure 1 B).

The effect of temperature on total soluble sugar concentration depended on the photoperiod length (significant interaction between temperature and photoperiod, Table 2), with sugar concentration increasing with Temp<sub>low</sub> and Temp<sub>high+low</sub> compared to Temp<sub>high</sub> only at the Photo<sub>short</sub> level (Figure 2B). For the Photo<sub>long</sub> treatment, sugar concentration remained high at all temperatures (Figure 2B).

The effect of temperature on final biomass also depended on the photoperiod during the acclimation period (significant interaction between temperature and photoperiod, Table 2); for the Temp<sub>low</sub> and Temp<sub>high+low</sub> treatments, Photo<sub>long</sub> increased biomass, while for the Temp<sub>high</sub> treatments, long photoperiod reduced biomass (Figure 3B). Under Photo<sub>long</sub>, temperature had no effect on flower production, while at Photo<sub>short</sub>, Temp<sub>high+low</sub> caused more plants to produce flowers than the Temp<sub>high</sub> and Temp<sub>low</sub> treatments (Figure 4).

**Table 2.** ANOVA summary table evaluating the effects of temperature (Temp<sub>low</sub>, Temp<sub>high+low</sub> and Temp<sub>high</sub>), photoperiod (Photo<sub>short</sub> and Photo<sub>long</sub>) and origin of *A. elatius* seeds (Sweden and Italy) on each of the response variables (height after acclimation and before frost, total soluble sugar concentration after acclimation and before frost, relative change in height and biomass). Northern and southern ecotypes were pooled within their countries of origin with each ecotype as a random factor (i.e. origin as fixed factor) Significant effects are set off bold.

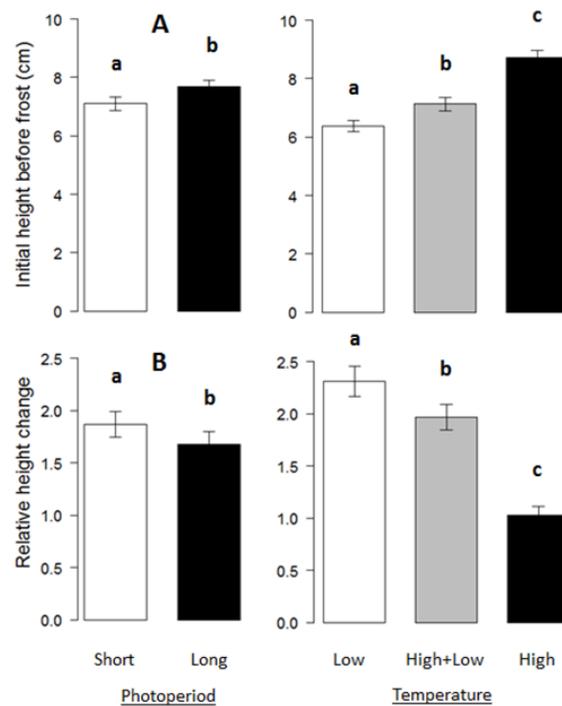
Height before frost	numDF	denDF	F-value	P value	Sugar concentration	numDF	denDF	F-value	P value
Origin	1	2	2.3	0.270	Origin	1	2	0.3	0.624
Temperature	2	89	33.3	<b>&lt;.001</b>	Temperature	2	87	3.3	<b>0.046</b>
Photoperiod	1	89	5.7	<b>0.019</b>	Photoperiod	1	87	27.3	<b>&lt;.001</b>
Origin *temperature	2	89	2.1	0.134	Origin *temperature	2	87	0.5	0.586
Origin *photoperiod	1	89	0.1	0.762	Origin *photoperiod	1	87	0.0	0.911
Temperature *photoperiod	2	89	0.6	0.542	Temperature *photoperiod	2	87	9.9	<b>&lt;.001</b>
Origin *temperature *photoperiod	2	89	1.9	0.151	Origin *temperature *photoperiod	2	87	0.8	0.471
<b>Relative height change</b>					<b>Biomass</b>				
Origin	1	2	8.2	0.104	Origin	1	2	4.7	0.162
Temperature	2	83	46.0	<b>&lt;.001</b>	Temperature	2	85	7.6	<b>0.001</b>
Photoperiod	1	83	6.3	<b>0.014</b>	Photoperiod	1	85	4.9	<b>0.029</b>
Origin *temperature	2	83	1.3	0.273	Origin *temperature	2	85	3.5	<b>0.034</b>
Origin *photoperiod	1	83	0.0	0.948	Origin *photoperiod	1	85	0.6	0.431
Temperature *photoperiod	2	83	0.3	0.750	Temperature *photoperiod	2	85	9.3	<b>&lt;.001</b>
Origin *temperature *photoperiod	2	83	1.9	0.153	Origin *temperature *photoperiod	2	85	1.5	0.238

### 3.2 Ecotypic differences

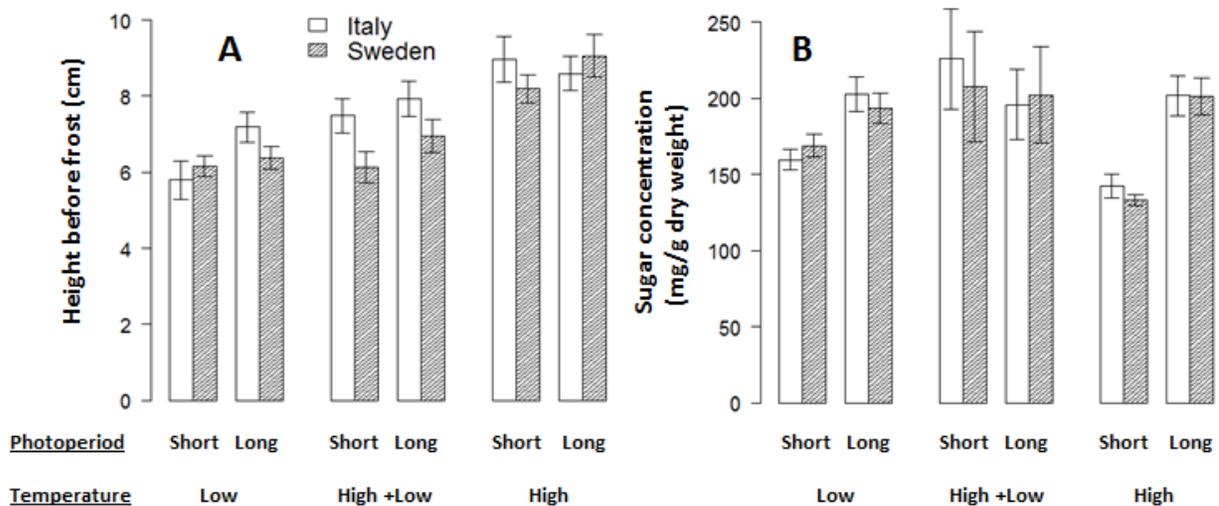
Southern ecotypes accumulated more biomass after Temp<sub>high+low</sub> than under other acclimation treatments, while for northern ecotypes there were no significant effects of treatment on biomass. (Table 2; Figure 3B). Plants from the two latitudes generally did not differ in their pre- and post-frost growth, as well as in total soluble sugar content (Table 2; Figure 2 A and B and Figure 3A).

### 3.3 Ecotypic variation in relative temperature and photoperiod sensitivities

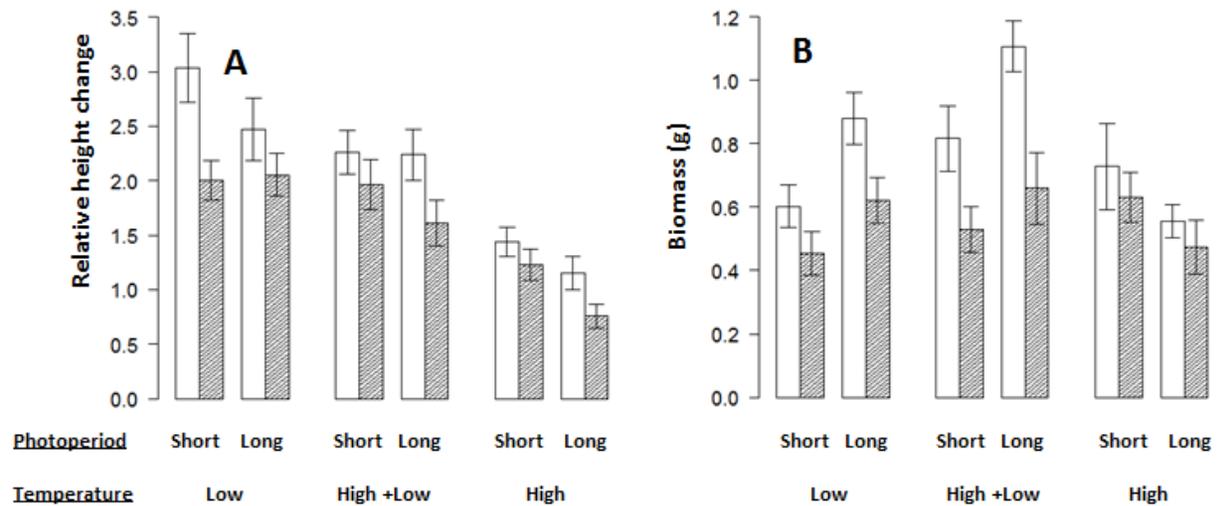
The northern and southern ecotypes exhibited differences in their relative responses to the treatments. Only the height before frost parameter showed significant ecotypic interactions with temperature and/or photoperiod (Table 3). For the relative temperature sensitivity (Figure 5 A), at Photo<sub>short</sub>, the southern ecotypes grew more than the northern ecotypes. The southern ecotypes were also more sensitive to photoperiod at low temperature than at high temperature (higher Photo<sub>long</sub> to Photo<sub>short</sub> at Temp<sub>low</sub> ratio in Figure 5 B), while the responses of the northern ecotypes to photoperiod were not as dependent on temperature.



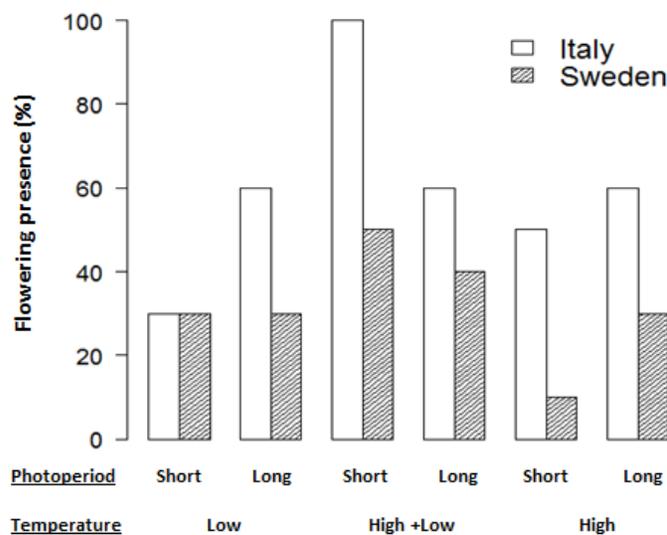
**Figure 1.** Overall acclimation effects of temperature and photoperiod on height before frost in pooled northern and southern *A. elatius* ecotypes. A: pre-frost height differences due to photoperiod and temperature effects across all temperature and both photoperiod levels, respectively. B: post-frost relative height change differences due to photoperiod and temperature effects across all temperature and both photoperiod levels, respectively. Relative height change: (final plant height – height before frost)/height before frost. Mean values ( $\pm$  1 standard error;  $n=60$  per photoperiod and  $n=40$  per temperature level) are shown. Significant differences ( $p= 0.05$ ) are marked different letters.



**Figure 2.** Acclimation effects before the frost event. Pre-frost height (A) and total soluble sugar accumulation responses (B) of northern and southern *A. elatius* plants after six different acclimation regimes, comprising of three temperature levels ( $Temp_{low}$ ,  $Temp_{high+low}$  and  $Temp_{high}$ ) and two photoperiod levels ( $Photo_{short}$  and  $Photo_{long}$ ) and before the frost manipulation. Mean values ( $\pm$  1 standard error;  $n=20$  per treatment) are shown.



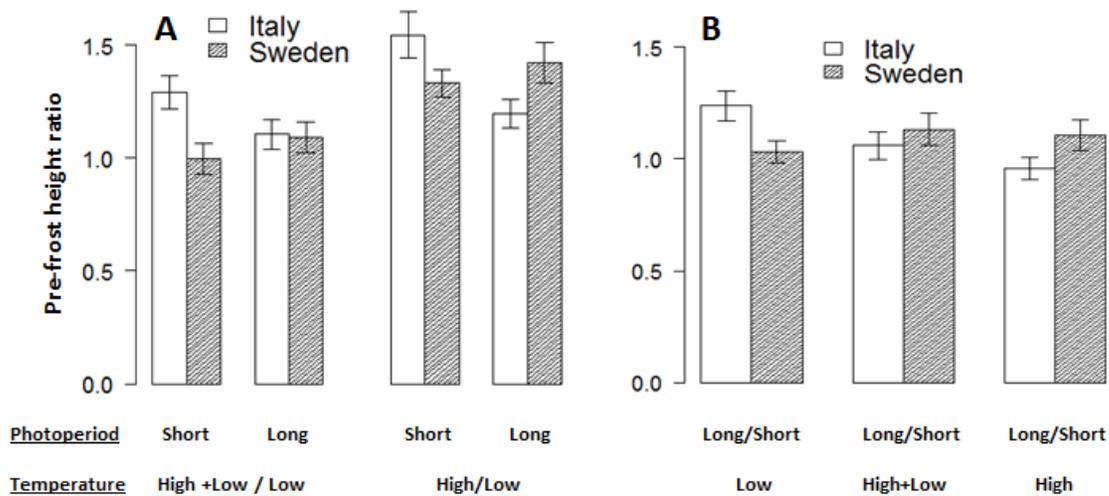
**Figure 3.** Relative height change after frost (A) and biomass (B) responses of northern and southern *A. elatius* plants in response to six different acclimation regimes, comprising of three temperature levels ( $Temp_{low}$ ,  $Temp_{high+low}$  and  $Temp_{high}$ ) and two photoperiod levels ( $Photo_{short}$  and  $Photo_{long}$ ) obtained after acclimation, a preceding frost event ( $-8^{\circ}C$ ) and growth for 5 weeks under optimal growing conditions. Mean mean values ( $\pm 1$  standard error;  $n=20$  per treatment) are shown. Relative height change was calculated as (final plant height - height before frost)/height before frost, where height before frost was measured after cold acclimation and final plant height was measured 4 weeks after the frost treatment. Biomass was measured once, 5 weeks after the frost treatment and represents the effect of both acclimation and frost on plant biomass.



**Figure 4.** Flower presence (% flowering individuals) obtained after five weeks of regrowth in northern and southern *A. elatius* ecotypes in response to six different acclimation regimes, comprising of three temperature levels ( $Temp_{low}$ ,  $Temp_{high+low}$  and  $Temp_{high}$ ) and two photoperiod levels ( $Photo_{short}$  and  $Photo_{long}$ ) obtained five weeks after a frost event ( $-8^{\circ}C$ ) ( $n=20$  per treatment).

**Table 3.** ANOVA summary table evaluating the relative effects of temperature ( $Temp_{high+low} / Temp_{low}$  and  $Temp_{high} / Temp_{low}$ ) and photoperiod ( $Photo_{long} / Photo_{short}$ ), and latitude of *A. elatius* seeds (Swedish and Italian origins) on the height prior to frost. northern and southern ecotypes were pooled within their latitude with each ecotype as a random factor (i.e. latitude as fixed factor). Of the relative response ratios for growth rate before frost, sugar concentration and biomass parameters, only significant ecotype-temperature /ecotype-photoperiod results are presented.

Relative height before frost (temp effect)	numDF	denDF	F-value	p value
Latitude	1	2	1.361	0.364
Temperature	1	53	27.416	<0.001
Photoperiod	1	53	3.445	0.069
Latitude × temperature	1	53	3.020	0.088
Latitude × photoperiod	1	53	13.243	<0.001
Temperature × photoperiod	1	53	0.834	0.365
Latitude × temperature × photoperiod	1	53	0.575	0.452
Relative height before frost (photoperiod effect)				
Latitude	1	2	0.311	0.876
Temperature	2	36	2.13	0.134
Latitude × temperature	2	36	4.917	0.013



**Figure 5.** Relative pre-frost height responses of northern and southern *A. elatius* ecotypes, evaluating **A**: relative temperature effect (ratios of plant height reached at warmer temperatures to plant height at colder temperature), expressed as the height ratios of  $Temp_{high+low}$  relative to  $Temp_{low}$  and  $Temp_{high}$  relative to  $Temp_{low}$  within each photoperiod ( $Photo_{short}$  and  $Photo_{long}$ ) and **B**: relative photoperiod effect (ratios of growth at  $Photo_{long}$  relative to growth at  $Photo_{short}$ ) within each temperature ( $Temp_{low}$ ,  $Temp_{high+low}$  and  $Temp_{high}$ ). Mean values (+/- 1 standard error; n=20 per treatment) are shown.

## 4. Discussion

### 4.1 Influence of photoperiod vs. temperature on pre and post-frost growth.

Both temperature and photoperiod influenced cold acclimation in *A. elatius*. In our experiment, a difference in mean acclimation temperature of 2 °C (from 5 °C to 3 °C) had a more positive effect on plant regrowth after a freezing stress than a difference in photoperiod of 6 h (from 12 to 6 h). Given that the maximum yearly photoperiod change encountered by the southern ecotypes is around 6 h, while the difference in mean temperature of the warmest and coldest annual quarters is around 13 C° (Table 2; Figure 1), temperature appeared to be a stronger trigger of cold acclimation than photoperiod in our experiment. Similarly, a difference in mean acclimation temperature of 5 °C (from 8 °C to 3 °C) had 5 times the effect of the photoperiod difference on post-frost growth. Climate change is expected to feature increased temperature variability (Schar et al., 2004), and unpredictable frost events are still expected to occur with unchanged magnitude, although with reduced frequency in the future (Kodra et al., 2011). Our results from a few temperature and photoperiod levels imply that at least our target species, *A. elatius*, may face increased frost damage in a warmer world. It remains to be seen, however, if the lesser role of photoperiod, which still was significant in influencing cold acclimation, suffices as a safety belt in the face of climate warming.

Our results imply a balance between greater growth prior to frost and weaker growth (i.e. more severe frost damage) after frost with warmer acclimation temperatures, which are linked with an extension of the fall growing period (Vitasse et al., 2009). In a similar experiment exploring relative temperature vs. photoperiod influence on cold acclimation, raspberry ecotypes were also more sensitive to temperature decreases (4 °C vs. 20 °C) than to changes in photoperiod (9 h vs. 18 h), although the temperature difference relative to photoperiod difference was greater than in our experiment (Palonen, 2006). The high relative temperature effect on pre and post-frost growth also supports the finding that a short photoperiod initiates growth cessation mainly at high temperatures (Weiser, 1970), whereas decreasing temperature has the strongest influence on cold acclimation (Junttila, 1996). However, experiments comparing the relative effects of temperature vs. photoperiod cues must be interpreted carefully in the context of the treatment levels selected, and whether they represent meaningful variation in these factors. In our

study, the two photoperiod treatments differed by 6 h, which corresponded to the maximum yearly photoperiod change encountered by the southern ecotypes (15.5 h max., 9 h min.), while the northern ecotypes experience the same photoperiod change from late September to November (20 h max, 5 h min). Despite the minimum 6 h photoperiod being almost 3 h lower than the minimum photoperiod experienced by the southern ecotypes, the plants still grew as well as the southern ecotypes, not showing a severe photoperiod growth limitation. The treatment levels for acclimation temperature were chosen to match values below, at and above 5°C, which is the typical threshold for the initiation of strong increases in cold acclimation (Paquin and Pelletier, 1980).

#### 4.2 Variation in biomass responses among ecotypes.

Surprisingly, the southern *A. elatius* ecotypes exhibited the highest post-frost biomass in response to the intermediate, Temp<sub>high+low</sub> treatment, whereas we expected the highest degree of acclimation, and thus the most post-frost growth, in response to Temp<sub>low</sub> acclimation, in the northern ecotypes. The plants under Temp<sub>high+low</sub> treatment were apparently able to take advantage of favourable growth conditions during the acclimation phase, yet also received sufficient acclimation cues to avoid substantial frost damage. Both total biomass and flower production were favoured by this treatment for the southern ecotypes. In contrast, the Temp<sub>high</sub> treatment likely permitted too much frost damage by providing too weak of a cold acclimation cue, whereas the Temp<sub>low</sub> treatment provided a strong cold acclimation cue, but at the expense of slow pre-frost growth. Therefore, we propose that plant growth rate prior to frost events may also be an important determinant, in addition to achieving sufficient cold acclimation, in achieving the highest biomass during the growing season. Likewise, the timing of plant senescence in the fall can affect growth in the following year by affecting nutrient remobilization and photosynthate storage (Lim et al., 2007). Late fall plant senescence can increase photosynthate storage, but can also result in improper nutrient remobilization due to fall frost damage, because of insufficient fall cold acclimation (Keskitalo et al., 2005). Therefore, ecotypes which grow longer into the fall may also grow faster in the growing season, as long as the frost damage is not severe. In our case, the higher accumulated fall biomass in the southern ecotypes can possibly be related to the resumption in fall growth commonly observed for cool-season (C<sub>3</sub>) grasses growing in areas

where high summer temperatures and drought conditions cause a temporary cessation in growth (Hutchison and Henry, 2010).

#### 4.3 Differences in relative temperature and photoperiod sensitivities between northern and southern ecotypes.

The relative temperature and photoperiod sensitivity analyses (Figure 5; Table 3) allowed us to assess the extent to which northern ecotypes were able to cold-acclimate relative to the southern ecotypes at the different temperature and photoperiod levels. The southern ecotypes grew more at Temp<sub>high</sub> at Photo<sub>short</sub> than the northern ecotypes, showing that northern ecotypes may cold acclimate to a higher extent at Photo<sub>short</sub>. Southern tree ecotypes also require shorter photoperiods to achieve similar cold acclimation levels as the northern tree ecotypes (Howe et al., 1995; Li et al., 2003), the latter being more responsive to shorter photoperiod and temperature (Li et al., 2005). Latitudinal photoperiod sensitivities in our grass species therefore appear similar to the pattern observed for trees. In trees, northern ecotypes have typically been more responsive to changes in photoperiod and temperature than southern ecotypes, with the latter being able to maintain growth at lower temperatures as a result. (Junttila, 1982; Howe et al., 1995; Li et al., 2002; Junttila et al., 2003). Our results imply that the overall cold acclimation pattern is similar in tree and grass species. Our results further show that latitudinally separated grass ecotypes are likely to differ in their biomass responses to the cold acclimation conditions that precede frost.

#### 4.4 Total water soluble sugar concentration as a proxy for frost hardiness.

Plant tissue soluble sugar content normally increases with decreasing temperature and photoperiod as part of the cold acclimation process, because it maintains cell membrane integrity during freezing and thawing (Strimbeck et al., 2008; Hanslin and Hoglind, 2009; Ostrem et al., 2010). Total soluble sugar concentrations did not differ between northern and southern ecotypes in our study, so it is possible that other cold acclimation strategies, such as increased anti-freeze protein formation (Antikainen and Griffith, 1997), as well as increases in dehydrin proteins (which stabilize proteins and cell membranes during cell dehydration; Kosova et al., 2007), may play a more important role in cold acclimation than sugar accumulation in this species. Similarly, no clear relationship between water soluble carbohydrates and winter hardiness exists for other

grass species (Lawrence et al., 1973; Livingston and Premakumar, 2002). Individual sugar types may need to be quantified, in addition to the total sugar content, because species specific increases in particular sugar types may be more directly related to cold acclimation (Sandrin et al., 2006). It is also possible that the difference in our temperature treatments was simply too subtle to create detectable differences in sugar concentrations. Our experiment featured a 5 °C temperature difference between suboptimal growing temperatures, so only minor differences in sugar tissue content was to be expected (i.e. for temperatures close to the cold acclimation optimum (below 5 °C), sugar concentration may be a less accurate evaluation of cold acclimation level, as compared with growth performance). On the other hand, water soluble high molecular weight fructans, that had not been taken into account in our sugar quantification, could have contributed to the freezing tolerance of *A. elatius*. For instance, when comparing the freezing tolerance of 42 ecotypes of *Poa annua* L., Dionne et al. (2010) found a significant correlation between the concentration of high molecular weight fructans in cold-acclimated ecotypes and their freezing tolerance. Another potential confounding factor that may explain the high sugar concentration observed for all temperature treatments at Photo<sub>long</sub> is that high light can promote increased sugar concentration, independent of cold acclimation, by promoting high daily rates of photosynthesis (Eckardt et al., 1997). Thus, for temperate grasses, soluble sugar concentration may indicate a balance between photosynthesis and growth rather than cold acclimation level, and the relationship between frost tolerance and soluble sugar concentration relationship may be genotype specific (Pollock et al., 1988).

#### 4.5 Conclusions

Experimental acclimation temperatures were more important for the cold acclimation of the common temperate grass species *A. elatius* than photoperiod. This finding suggests that climate warming could enhance fall productivity at the expense of cold hardening. Based on this result, frost damage could become more common in a warmer, yet also more variable, climate of the future. Increased growth in fall, however, also positively affected total biomass production despite concomitantly increasing frost damage in our study. The lower biomass production of northern ecotypes was potentially caused by quicker acclimation to cold acclimation cues, which led to reduced frost damage in comparison to southern ecotypes, yet also to reduced total biomass production. Despite being more prone to frost damage, the southern ecotypes outperformed the

northern ecotypes under the environmental conditions of our study due to a greater accumulation of biomass during the growing and the acclimation periods.

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## **Manuscript 3: Common garden experiments to characterize cold acclimation responses in plants from different climatic regions**

Book chapter in Plant Cold Acclimation (Methods in Molecular Biology), Published

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### **Summary**

Cold acclimation is a crucial factor to consider with ongoing climate change. Maladaptation with regard to frost damage and use of the growing season may occur, depending on cold acclimation cues. Importance of photoperiod and preceding temperatures as cues needs therefore to be evaluated within (ecotypes) and among species. Common garden designs, in particular the (1) establishment of multiple common gardens along latitudinal/altitudinal gradients, (2) with in-situ additional climate manipulations and (3) with manipulations in climate chambers are proposed as tools for the detection of local adaptations and relative importance of temperature and photoperiod as cues for cold adaptation. Here, we discuss issues in species and ecotype selection, establishment of common gardens including manipulations to temperature and photoperiod, and quantification of cold adaptation.

**Key words:** common garden experiments, provenance trials, cold acclimation, frost tolerance, experimental design, within-species variability, intra-specific variability

### **1. Introduction**

Cold acclimation (also known as hardening) is a suite of changes in gene expression and physiology that increases plant tolerance to cold temperatures (1, 2). In the fall a reduced photoperiod and declining temperatures trigger cold acclimation in perennial plants (3, 4). The topic of plant winter acclimation has received increasing attention in the context of global climate change, because the latter is expected to increase temperature variability. Highly fluctuating

temperatures can disrupt plant cold acclimation, making plants less hardy to withstand unexpected frosts, which are predicted to occur with prior magnitude despite decreased frequency (5).

The relative importance of temperature and photoperiod may be critical for determining how effectively plants acclimate to prevent frost damage in a changing climate. Plants that use temperature as the main acclimation cue can benefit from a longer growing season, but can also be more susceptible to unexpected frosts. On the other hand, plants that respond strongly to photoperiod may be less susceptible to unexpected frosts. Cold acclimation cues have been studied most intensively in trees, for which there is high interspecific variation in the dominance of temperature versus photoperiod in driving cold acclimation responses (6). In temperate regions, woody plants cold acclimate to a greater extent than herbaceous species, likely because the latter can be insulated from cold air temperatures by snow cover (7). Snow cover is decreasing substantially in some regions (8, 9), which can increase the exposure of herbaceous plants to cold temperatures over winter, despite an overall increase in mean air temperatures. Therefore, herbaceous plants are an important contrasting functional group to woody plants in the study of cold acclimation responses to climate change.

Frost susceptibility also varies substantially among ecotypes within species. An ecotype is a general term used to describe plants within a species that display genetic differentiation to particular environmental parameters within the ecotype's habitat (10, 11). Ecotypes at lower latitudes are generally less cold tolerant than ecotypes at higher latitudes (12). Species distributions can span across continents and along great elevational gradients. Local adaptation creates ecotypes that may differ as much in their acclimation and freezing tolerance as different plant species (13). Thus, knowledge of local adaptation is needed to more precisely predict species' climate change responses. In addition, because of the potential for long distance dispersal and population range shifts, ecotypes of different species from different climatic regions need to be incorporated into cold acclimation studies in order to predict regional changes in species composition. Relating cold hardening responses to local environmental conditions will also improve the understanding of cold hardening cues in general.

Common garden experiments involve the transplantation of experimental subjects into a common growth environment before taking measurements (14), and allowing plant responses to be compared under standard environment conditions, with control over factors such as ontogenetic stages. Measurements of ecotypes in their natural environment along a spatial or temporal climatic gradient is an alternative approach that can be used to study cold acclimation responses (14), but it includes plant-plant interactions which are minimised in common garden experiments and is beyond the scope of this chapter. Although common garden experiments can be performed under naturally variable weather conditions, the addition of controlled environmental conditions (e.g. variation in photoperiod / frost intensity) allows for a better mechanistic understanding of plant responses. Here we describe the set up of common garden experiments designed to test cold acclimation responses, performed with and without additional manipulations. First, experimental guidelines are presented that apply to common garden experiments without additional environmental manipulation. Implementing additional environmental manipulations are then discussed and finally potential response parameters for such experiments are presented. An overview of the step-wise procedure involved in the set up and implementation of cold acclimation experiments on plants from different climatic regions is presented in Figure 1.

The guidelines are intended to be general, and to apply to most vascular plants. The actual experimental design needs will likely require modification based on the specific species used, taking factors such as plant size and seed production into consideration.

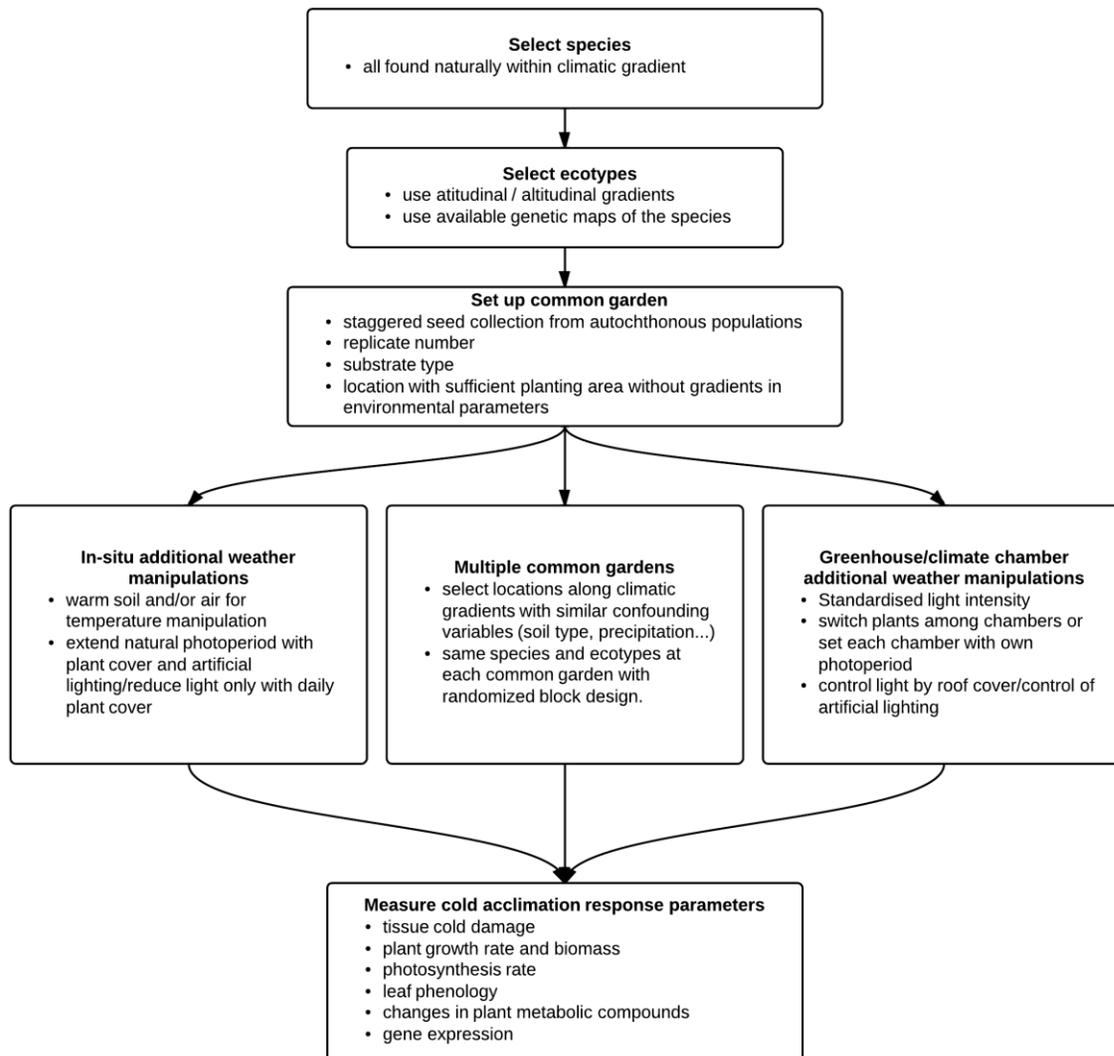
## **2. Material**

### **2.1. Ecotype selection**

After choosing the target plant species, ecotypes from each species need to be carefully selected in the context of the research question. Altitudinal and latitudinal gradients are typically chosen to select ecotypes (14, 15). To aid with ecotype selection, a species distribution map can be overlaid on a climatic map. For example, minimum winter temperature and annual photoperiod changes within the species range can be used as proxies for climate adaptation and ecotype selection. Most commonly, latitude has been taken as a proxy for climate, because it correlates with many biologically relevant environmental gradients, including photoperiod (16).

Existing databases such as Worldclim database ([www.worldclim.org](http://www.worldclim.org)) (17), as well as photoperiod (18) can be used to acquire such data.

**Figure 1.** Steps required to carry out cold acclimation experiments on plants from different climatic regions (different ecotypes) in one or more common gardens, with or without additional weather manipulations (see Table 1 for respective treatment comparisons). Bullet points highlight factors to consider in each step.



Altitudinal gradients allow an isolation of temperature adaptation, with greater changes in temperature per distance than latitudinal gradients (19), while latitudinal gradients allow the combined effects of photoperiod and temperature to be considered. If the latitudinal species distribution effect on acclimation is of prime interest, the elevation of the ecotype origins should be as equal as possible to avoid confounding environmental factors. In both cases, care should be exercised in selecting ecotypes that stem from locations differing as little as possible in confounding factors not under study, such as precipitation and soil type (15, 20). There also exist statistical methods to control for confounding factors (see Data analysis section). Cold tolerance is commonly used to describe the extent of the achieved cold acclimation. To maximize the likelihood of selecting ecotypes that differ most with respect to cold tolerance, the longest possible gradient along the distribution of the species range should be used. It is advisable to select at least two distinct ecotypes (separated enough to limit cross pollination) in relatively close proximity to each other for each latitudinal/altitudinal point of origin, in order to increase the confidence that the variation in differences is due to the latitudinal position, rather than to specific local conditions.

Differences in geographically influenced acclimation trends result from genetic adaptation or from epigenetic modifications (plastic plant responses) (21). Climatic influence on the genetic adaptation of ecotypes is well known (22). Available genetic data of plant populations across the distribution range of a species can thus be considered for selecting the most genetically distinct ecotypes, and genetic differences can be compared to differences in the acclimation responses upon completion of the experiment (for an example of genetic analysis *see* (23), and for an example of climatic origin vs. cold hardiness comparison *see* (24).

## **2. Establishment of a common garden**

### **2.2. Seed collection and plant numbers**

Ecotypes originating from different latitudes flower and produce seed at different times, which necessitates staggered timing of seed collection along the latitudinal gradient. Ideally, single clones or mother plants need to be sampled individually from autochthonous populations and kept separated throughout germination trials. If variation among individuals within each ecotype is to be included in the analysis, at least five different clones or mother plants per

ecotype should be used as seed sources, with at least five replicate plants being grown per mother. Especially for clonal plants it needs to be assured that the mother plants do not stem from the same clone, by taking into account the distance typically reached by rhizome growth and dispersal potential of detached propagules. Without analysing mother plant response variability, the replicate number depends on the number of ecotypes being compared. Ideally, plant traits should be measured for the ecotype to be used in the experiment to gauge natural variability, which will help the right replicate number to be selected effectively. Examples below are given as a reference:

- Ten replicates were used for three ecotypes, both in determining the relationship of differences in allele frequencies at polymorphic traits to phenotypic plant traits (25), and in quantifying autumn cold tolerance (21).
- Five trees were sampled per species, using 14 species in total for bud burst as a function of photoperiod (6).
- Ten plants per ecotype with a total of ten grass ecotypes were used in a frost tolerance experiment (26).
- Four replicate raspberry plants per cultivar with a total of six cultivars were used to monitor growth at different cold acclimation temperatures (27).
- 

### **2.3. Plant propagation**

After seed collection, germination and propagation methods should be standardised across all ecotypes, with seedlings being first potted or planted in a standard substrate in a common garden. Pot size depends on the duration of time the plants need to remain potted. If space is limited, deeper rather than wider pots are preferred to ensure roots do not become pot bound. Once roots reach the bottom of the pot, acclimation temperature should be lowered to 5°C or below, which causes roots to cease growth (28). Although root growth is species-specific, roots in three month old juniper cuttings have been shown to grow 0.2 mm per day at 6°C, as compared with 1 mm per day at 15°C (29). Roots can also be trimmed and repotted to promote regular growth (30).

Substrate type can influence the rate of root acclimation as a result of differences in heat conducting ability and water holding capacity, with higher water moisture content reducing the

freezing and thawing rates (31). A major deciding factor in substrate selection is whether the roots will need to be washed and analysed after the conclusion of the experiment. Proportionally higher sand content allows easier root washing while minimizing root damage. The drawback of a high sand substrate is that it has low water and nutrient holding capacities (31). Frequent watering and nutrient addition (e.g. Hoagland's solution) can be used to offset this problem. Additionally, sandier soils cause 1) faster root development (32), which can effect cold hardiness by altering root distribution within the substrate, and 2) reduce insulation of the roots (33).

Fertilization should be done with care, because the effects of fall nitrogen application can increase or decrease cold tolerance depending on the stage of cold acclimation at which it is added, as well as the particular species involve (34, 35, 36, 37, 38).

The chosen substrate at the experimental location should be standardised and characterised to the depth reached by the respective plant species. For long term common gardens (spanning multiple years), the distance between trees should be at least three meters for plants lower than 15 m in height and increased by a minimum of a meter for every additional 15 m of height. Standard planting measures based on the spacing of plants are specified by the International Phenological Gardens in Europe.

(<http://www.arm.ac.uk/nci/docs/Instructions-IPG.pdf>) (39).

### **3. Methods**

#### **3.1. Applying cold acclimation treatments**

For all types of treatments spatiotemporal replication of experiments is suggested to increase the confidence in results (14). A comparison of the three ways in which cold acclimation treatments can be administered is presented in Table 1.

**Table 1.** Comparison of cold acclimation treatments in common garden experiments to evaluate cold acclimation differences among plant species and ecotypes.

<b>Methodology</b>	<b>Best suited for</b>	<b>Advantages</b>	<b>Disadvantages</b>
1) <i>Common garden experiments without additional climate manipulation – replicated common gardens along a latitudinal/altitudinal gradient</i>	Disentangling photoperiodic and temperature cues for cold acclimation, replicating common gardens simultaneously along latitudinal and altitudinal gradients	- simulates most probable future climate scenario by incorporating most environmental variables of the new location - “hands off” experimental approach	- presence of confounding factors (e.g., wind patterns/precipitation sunlight) makes attributing differences to specific factors (photoperiod/temperature) difficult = “black box” experimental design - results depend on actual climatic conditions during the experiment at the single sites while sites were chosen due to their long-term climatic differences
<b>Methodology</b>	<b>Best suited for</b>	<b>Advantages</b>	<b>Disadvantages</b>
<i>Common garden experiments with additional climate manipulation treatments</i>			
2) <i>In-situ additional weather manipulations</i>	Mechanistic exploration of photoperiod/temperature effects on cold acclimation in a natural environment.	- more realistic than # 1 - limited control of temperature and photoperiod adjustments - less space/logistical constraints (larger/more plants possible)	- hard to replicate experimental set up and compare results to previous experiments due to high inter and intra annual temperature variability.
3) <i>Manipulations in climate chambers</i>	Mechanistic exploration of threshold cold acclimation responses to photoperiod/temperature changes. General exploration of plant acclimation cues (e.g., comparing the rates of temperature & photoperiod decreases on plant acclimation)	- high level of control over treatments - can be replicated with high precision	- not as realistic as <i>in situ</i> photoperiod and temperature manipulations. - space/logistical constraints (smaller/fewer plants possible)

### 1. Establishment of multiple common gardens along a latitudinal/altitudinal gradient

First, species should be used which all naturally occur within the altitudinal/latitudinal gradient of interest. Replicated common gardens are then established in two or more locations along the selected gradient. Two or more ecotypes from every species, typically stemming from two opposite climatic extremes are planted in every common garden in a randomized block design, with species and ecotypes being randomly mixed within each block. Using reciprocal native soils is not advised due to uncontrolled bias (site  $\times$  ecotype  $\times$  soil source interaction) (40). For experiments where plants are only to be observed for one to two seasons, potted seedlings (see 2.3. on preventing pot bound roots) can be left in containers, placed in prepared nursery beds, and sand/soil can be used to fill the gaps among pots for insulation (as done for tree seedlings by (40) and for grass by (36)).

Additional weather manipulations can offer an increased number of cold acclimation scenarios within the same time frame, aiming at a more mechanistic understanding of temperature and photoperiod vs. ecotype-specific acclimation responses. Additional climate simulations can be administered either in the field (in-situ) or in enclosed climate controlled chambers/greenhouses.

### 2. Additional climate manipulations in-situ

A variety of fall light and temperature manipulations can be applied to the plants in the study of cold acclimation.

#### Examples of temperature manipulation:

Overhead heating lamps to warm the plants with infrared radiation (13, 41).

Buried heating wires to increase soil temperature (42).

Open top passive warming chambers that increase temperature via decreased air flow and greenhouse effect (43).

#### Examples of light manipulation:

Light tight aluminium boxes in the field that close daily via a remote control (44).

Opaque plastic is wrapped around plants with additional incandescent light inside the covered units (45).

### 3. Manipulations in climate chambers

Greenhouses and small climate chambers can also be used to control temperature and photoperiod. In addition to computer controlled daily photoperiodic cycles, extension of natural photoperiod in greenhouses with artificial light can be used (46) or exposure to natural light can be decreased by automatically closing greenhouse roof panels (30). Two strategies can be used to impose different photoperiods: 1) different photoperiod cycles can be run in different chambers, while keeping temperature constant or 2) the same temperature can be maintained in two chambers with light being always on in one and off in another, switching plants between the chambers twice daily.

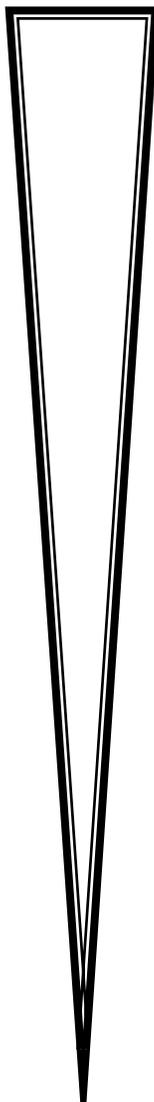
The first method requires less maintenance while the experiment is running, but it suffers from pseudoreplication, because no two chambers are alike, and potential chamber effects are confounded with the desired treatment differences. To circumvent this shortcoming, chamber settings can be switched at set time intervals to ensure plants spend equal time periods in all chambers, and to ensure the chamber effect is as equal as possible for all plants. This method is advised when multiple combinations of temperature  $\times$  photoperiod factors are used and when there is little mean variability in temperature and photoperiod among chambers. Treatments that differ in acclimating temperature will cause plants to lose water at different rates, and the bigger the temperature difference among treatments, the more often the plants should be watered to minimize soil moisture differences.

In the second method, in order to impose progressively lower photoperiod treatments, sets of plants are transferred twice daily at staggered intervals from one chamber to another to shorten or lengthen the effective photoperiod. This method allows for several photoperiod manipulations using only two climate chambers, while minimizing the chamber effect by all plants sharing time in both chambers, as compared to using a separate chamber for every different photoperiod. Periodically the no light and light chambers can be reversed to ensure the same time period is spent by all plants in both chambers.

#### 4. Response parameters

Table 2 presents plant parameters that can be used to quantify plant cold acclimation responses. Ultimately, the nature of the study will dictate the types of parameters that are to be measured. Functional responses attributable to responses of a plant as a whole have high ecological importance. Yet, they usually require natural or artificial frost events after acclimation in order to test for differences in cold tolerance. Ideally, replication is sufficient for a gradient of minimum temperatures to be tested. Imposing controlled frost events is even possible in remote field locations (47). Assessment of physiological pathways or plant responses at a molecular level improve mechanistical understanding of the processes involved and do not depend on actual frost events after acclimation.

**Table 2.** An overview of parameters that can be used to quantify plant cold acclimation responses. Whole plant functional responses are advised to be measured before more mechanistic response parameters.

Plant functional traits	Commonly measured parameter/s	Rationale	References for detailed explanation and methods	Level at which response parameter is measured
Fall phenology	- Leaf colour - Leaf fall	Plant phenology is one of the most sensitive parameters to changes in photoperiod and temperature, making it an ideal cold acclimation parameter.	Phenological guide of the international phenological gardens(53)	<p>Whole plant, functional long term responses to cold acclimation</p>  <p>Mechanistic immediate responses to cold acclimation</p>
Plant growth	- Growth rate - - Biomass - Seed production after acclimation and exposure to frost.	Growth rate may be faster following cold stress and equalise among treatments with time. Reproductive output may be independent from growth performance.	Reproductive output – (36)	
Functional effect of root frost damage	- Root N uptake after acclimation and exposure to frost	Roots are exposed to a solution of isotopically labelled N solution and then washed, dried and ground before tissue <sup>15</sup> N content is determined with a mass spectrometer	(54)	
Extent of plant issue cold damage.	- Relative electrolyte leakage after acclimation	When plant cell membranes are cold damaged, they release are damaged ions into the apoplast. The conductivity of the solution containing the ions is then measured and is proportional to cold damage.	(55, 21)	
Plant photosynthetic activity	- Chlorophyll fluorescence after acclimation	Acclimation stage as well as amount of cold damage sustained can be assessed from the amount of photoinhibition of plant photosystems as well as rates of CO <sup>2</sup> production / O <sub>2</sub> consumption.	(56, 57, 58)	
Plant acclimation stage/stress level	- Compounds that change their concentration with cold acclimation to prepare plants to withstand sub-zero temperatures	Increase in soluble sugar content and abscisic acid and the formation of antifreeze proteins, as well as structural changes in cell membrane retard ice formation and keep the cell membranes fluid, allowing plants to maintain normal metabolism. Specific genes are also only expressed when plants cold acclimate.	<u>Proteome changes:</u> (59, 60, 61) <u>Sugar concentration:</u> (62) <u>Gene expression:</u> (63) <u>Abscisic acid:</u> (64) <u>Cell membrane structural changes:</u> (45)	

## 2.Data analysis

Differences among climate treatments and among ecotypes/species, and in particular interactions between both factors, are tested by applying two-factor Analysis of Variance (ANOVA). In the case of nested replicates, mixed models with the blocking factor assigned as a random effect can be applied (48). Preferable designs are replicated common garden experiments in conditions similar to the home-site of all ecotypes (49). Local adaptation of populations can be assessed using a regression between the relative performances of the ecotypes in the common garden experiment and their home site climate (e.g. winter minimum temperature) (24). To allow for adequate statistical power in the regression analysis, the number of ecotypes should exceed ten. Statistical techniques such as multiple regression, hierarchical or mixed-effect models, variation partitioning, or structural equation modeling (20, 50, 51, 52) can be used to correct for confounding factors.

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## **Manuscript 4: Plant Responses to climatic extremes: within-species variation equals among-species variation.**

Journal (Global Change Biology, Submitted)

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

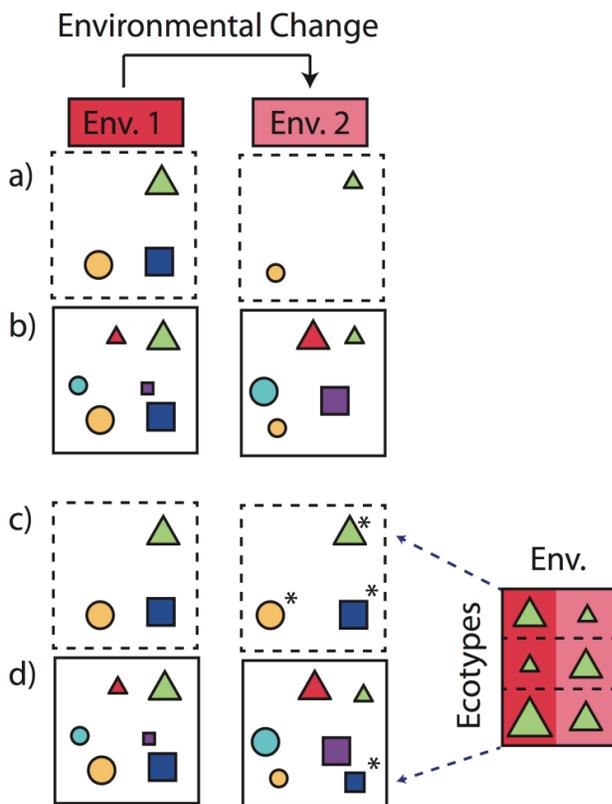
## ABSTRACT

Variation in stress responses among populations may have important implications for the prediction of changes in species distributions in response to climate change and general ecological theory; however, direct comparisons of among versus within-species variation in stress responses are lacking. We present a direct comparison of among versus within-species variation in response to two of the main stresses anticipated with climate change: drought and frost. We simulated 1) drought and warming and 2) spring frost for four common European grass species and their ecotypes collected from across Europe. To address how variation in responses among species from different functional groups compares to within-species variation, we also 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*. Response parameters included in the three respective experiments were: (1) C/N ratio and biomass (2) chlorophyll content and biomass and (3) plant greenness, root  $^{15}\text{N}$  uptake, and live and dead tissue mass. Using coefficients of variation (CV), a total of 77 within-vs among species comparisons were conducted, taking into account each experiment and each response parameter. 66 comparisons yielded no significant differences between within- and among-species variations in stress responses. Of the 11 significant effects, within-species CVs were higher than among-species CVs at single sites in six cases. For drought and warming as well as spring frost experiments within species variability (ecotypes) could explain an additional 6% of response variation after accounting for the among-species variation. Within-species variation being generally as high as among-species variation emphasizes the importance of including both within and among species variability in ecological theory (e.g. insurance hypothesis) and applications (e.g. species distribution models or 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 states that 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). Nevertheless, within-species genetic and phenotypic variation also can be high, and at times equal to among-species variation (Hughes *et al.* 2008; Poirier *et al.* 2012).



**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 ecosystems functioning can be maintained despite environmental change as functionally redundant but less adapted species may become important with environmental change. They might replace other species and take over their role in the system. However, as within-species variability of stress tolerance may be as high as the among-species, negative effects of environmental change may be buffered by (active, human induced or passive natural) introduction or natural presence of better adapted ecotypes. This is particularly important in species-poor communities (c) and less in species-rich ones, 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.

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). 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).

Variation in local adaptation also could 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 outcomes than when species are treated as uniformly responding units (Oney *et al.* 2013; Valladares *et al.* 2014).

Despite the emerging importance of extreme weather events as a key component of climate change impacts (Jentsch *et al.* 2007), empirical data comparing within-species variation in responses of these events to variation among species are lacking. Increasing climatic variability is expected to increase the frequency of severe heatwaves 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,

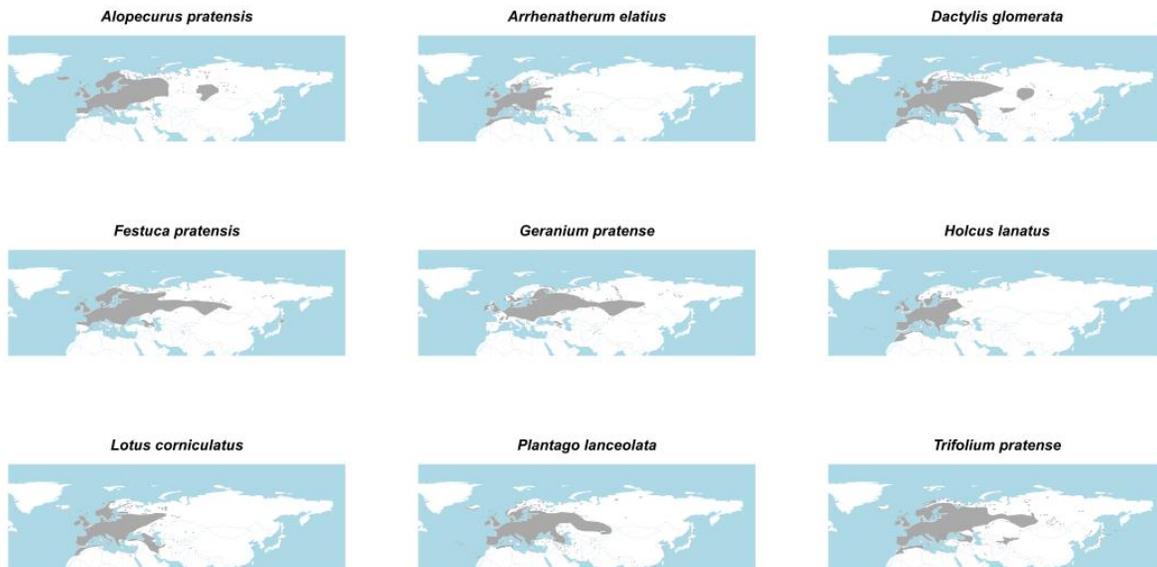
despite a general air warming trend; this increased risk is expected to occur because the timing of late frost is expected to remain relatively stable (Augspurger 2013), and the intensity and duration of frost events may not decrease within this century (Kodra *et al.* 2011). Furthermore, in the winter, 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 Giehl & Jarenkow 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 plant stress responses to climate change, and they encompass most temperature related stresses faced by plants.

Common European grass species express strong local adaptations to their climates of origin (Beierkuhnlein *et al.* 2011; Kreyling *et al.* 2012), and large-scale genetic gradients have been detected for species such as *Arrhenatherum elatius*, which is a wide-spread and abundant grass species in Europe (Michalski *et al.* 2010). We compared variation in stress tolerance among and within species by exposing the ecotypes of four common European grass species stemming from five European countries to simulated summer drought and warming as well as spring frost. We also 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. Overall, we hypothesized that variation in within-species responses to drought and warming, spring frost and frost after winter warm spells would vary as much as variation in responses among species from different plant functional groups.

## **MATERIALS AND METHODS**

### Species and ecotype selection

Natural distribution and mean climate parameters of all species used in all experiments are given in Fig. 2 and Table 1, 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.



**Figure 2.** 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).

**Table 1.** 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	Annual mean temperature (°C)	Mean maximum temperature (°C)	Mean minimum temperature (°C)	Annual precipitation (mm)
<i>Alopecurus pratensis</i>	4.0 ± 4.6	22.6 ± 3.6	-13.0 ± 8.1	639 ± 219
<i>Arrhenatherum elatius</i>	8.3 ± 3.5	23.9 ± 3.8	-5.4 ± 5.3	684 ± 206
<i>Dactylis glomerata</i>	5.8 ± 4.8	24.2 ± 4.2	-11.0 ± 7.9	614 ± 219
<i>Festuca pratensis</i>	3.8 ± 4.1	23.1 ± 2.8	-14.1 ± 8.1	612 ± 209
<i>Geranium pratense</i>	3.0 ± 3.9	23.1 ± 2.4	-15.7 ± 7.9	579 ± 154
<i>Holcus lanatus</i>	8.0 ± 4.0	23.7 ± 4.4	-5.8 ± 5.6	698 ± 256
<i>Lotus corniculatus</i>	7.1 ± 4.4	24.0 ± 4.4	-8.2 ± 6.7	661 ± 244
<i>Plantago lanceolata</i>	6.0 ± 4.1	24.4 ± 4.0	-10.9 ± 7.5	600 ± 246
<i>Trifolium pratense</i>	4.3 ± 4.7	23.4 ± 3.7	-13.3 ± 8.4	608 ± 209

The experiments took place in summer 2009 and spring 2010, respectively, with four grasses from Central European managed grasslands (*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 (assuming adaptation to local climate) from climatically 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.

For the winter warming plus frost experiment, within-species variation was represented by 11 genetically distinct ecotypes of *A. elatius*, selected from four European countries, using genetic data from Michalski *et al.* (2010) (Table 2). For this species, there is evidence of local adaptation in biomass production after spring frost at the continental scale (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). 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 group. This ensured that the inherent local variation of *A. elatius* was also accounted for in the analysis of among-species variation.

#### Drought and warming experiment

The common garden experiment was established in March 2009 in Bayreuth, Germany (49°55'19" N, 11°34'55" E). Grass ecotypes were cultivated in Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Poel, Germany, from February to April 2009 and delivered to Bayreuth for the start of experiment in May 2009 (see Beierkuhnlein *et al.* 2011 for site climate, plant cultivation and experiment details).

**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	Country	Mean maximum temperature of warmest month (°C)	Latitude	Longitude	Elevation (m a.s.l.)	Mean minimum temperature of coldest month (°C)	Annual precipitation (mm)
<b>Species and ecotypes used in drought and warming experiment and in spring frost experiment</b>							
<i>Arrhenatherum elatius</i>	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
<i>Alopecurus pratensis</i>	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
	Bulgaria	25.5	42°19'N	23°45'E	710	-4.9	585
<i>Festuca pratensis</i>	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
	Bulgaria	27.7	42°31'N	24°48'E	330	-4.5	581
<i>Holcus lanatus</i>	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
<b>Species and ecotypes used in winter warming plus frost experiment</b>							
<i>Arrhenatherum elatius</i> (Within-species diversity category)	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
	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
	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	
<i>A. pratensis</i> , <i>H. lanatus</i> , <i>A. pratensis</i> , <i>G. pratense</i> , <i>P. lanceolata</i> , <i>L. corniculatus</i> , <i>T. pratense</i> , (Among-species diversity category)	Germany	17.7	49°10'N	9°34'E	460	-2.5	676

The climate manipulations were performed twice, whereby the drought lasted 16-19 days in 2009, depending on the species-specific tolerance (drought ended when two-thirds of the individuals of one species showed severe senescence, see (Beierkuhnlein *et al.* 2011), and 30

days for all species in 2010. Analyses of combined data are presented in this paper. The drought treatments (control vs. drought) were combined with warming treatments (control vs. warming) in a split-plot design. The two climate treatments were fully crossed, resulting in four climate manipulations (control, drought, warming, and warming combined with drought), which were replicated three times, i.e. 12 experimental units in total. Each ecotype was replicated with seven plants per experimental unit (nested replicates). The available plants were assigned randomly to the 12 experimental units for each species. Each experimental unit was covered by a single rain-out shelter with an edge height of 80 cm, transmitting nearly 90% of photosynthetically active radiation.

The control precipitation consisted of biweekly watering with rain water, with the magnitude based on local daily 30-yr average precipitation. The annually recurrent pulse drought consisted of a period without precipitation and then rewetting the plants by the same amount of precipitation which was removed during the pulse drought manipulation. Warming was performed passively for the whole experiment via wind-shelters and black floor-covers, (average temperature increase of 1.4 K compared with the temperature control).

Above-ground biomass was harvested end of June and end of September in 2009 and 2010. Leaf C and N concentrations were measured in 2009 after the first drought. Samples were oven dried for 24 h at 80 °C, then fine-milled. Samples (approx. 3 mg) were analyzed using an elemental analyzer (EA 3000; Euro Vector, Italy). Leaf C and N concentrations (g/plant) was calculated from this analysis.

### Spring frost experiment

This experiment was conducted on the same plants used in the 2009 drought experiment (Beierkuhnlein *et al.* 2011), with three replicates per factorial group of ecotype and pretreatment (control, drought, warming, drought and warming) exposed to a late frost event in the night from 26 to 27 of May 2010 and another three replicates per factorial group of ecotype and pretreatment used as a control. Details on the experiment can be found in Kreyling *et al.* (2012).

From 2009 to 2010, the plants were overwintered in a sand-bed and exposed to ambient conditions before being exposed to a late frost event in the night from 26-27 May 2010. No late frost event occurred naturally in 2010. Based on local climate data, a late frost event of  $-5\text{ }^{\circ}\text{C}$  was simulated for three hours by gradual cooling inside a cooler truck.

The temporal pattern of chlorophyll content was monitored weekly for five weeks after the late frost manipulation using a SPAD-502 chlorophyll meter (Konica Minolta Sensing) on four randomly chosen leaves per plant. SPAD-readings were calibrated to foliar chlorophyll content for 20 leaves per species, resulting in 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.

#### Winter warming plus frost experiment

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  $\times$  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  $6.4\text{ }^{\circ}\text{C}$  and  $20.0\text{ }^{\circ}\text{C}$ , respectively. Light was provided with 400-W lamps (approximately  $600\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ ), 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  $10\text{ }^{\circ}\text{C}$  and  $6\text{ }^{\circ}\text{C}$ , respectively, photoperiod was decreased to 9 h, and light intensity was  $200\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ . To complete plant cold acclimation, the photoperiod was lowered to 8 h for one month, with soil surface temperature averaging  $0.0\text{ }^{\circ}\text{C}$  (minimum  $-6.2\text{ }^{\circ}\text{C}$ ; maximum  $+5.8\text{ }^{\circ}\text{C}$ ). Plants were kept at  $-1.5\text{ }^{\circ}\text{C}$  prior to thaw treatments, which took place 12-23 February 2012.

On 12 February all plants (10 plants per ecotype and species per treatment) were assigned to one of three thaw treatments: 12 h at  $4\text{ }^{\circ}\text{C}$  (mild thaw), 2 days at  $9\text{ }^{\circ}\text{C}$  (moderate thaw) or 6

days at 9 °C (extended thaw). 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. Due to technical problems, the minimum temperature was lowest for the frost in the mild thaw group, i.e. the group for which we expected the least frost damage. 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.

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 was 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  $^{15}\text{N}$  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  $\mu\text{M}$   $^{15}\text{NH}_3^{15}\text{NO}_3$  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  $\text{CaCl}_2$ , 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). No leaf N uptake was quantified for the mild thaw treatment.

## 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, <sup>15</sup>N uptake). For drought and warming experiment the model was “response ~ species\*drought\*warming + country\*drought\*warming”. For the spring frost experiment the model was “response ~ species\*frost + country\*frost”. Replication and experimental unit (plant location) were used as random factors for both experiments. For biomass analysis of drought and warming experiment, total biomass per year was used, with year as a random factor.

For winter warming plus frost experiment one model was used for species\*treatment interaction and another for ecotype\*treatment interaction to show that both species and ecotypes had similar interactions with treatments. For the overall treatment effects treatment levels comprised the fixed factor while the species- and ecotype-identities were inserted as a random factor in order to account for missing independence within these groups. 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*

Here, we aimed at the comparison of variation in stress responses within and among species using coefficients of variations (CVs), similar to (Jung *et al.* 2010). Therefore, 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. CVs were then used to detect differences between within- and among-species variations by linear mixed effect models with  $n =$  the number of species / ecotypes by linear models ( $CV \sim$  within / among, with treatment as a

random variable). For drought and warming as well as for spring frost experiments within- vs among-species comparisons were made using CVs incorporating all treatments while for the winter warming plus frost experiment, the within- vs. among-species comparisons were run separately for each treatment. In each country-specific within- vs. among-species comparison the local ecotype of each species was included in among but not in within-species variation to avoid data repetition.

For drought and warming as well as spring frost experiments variance partitioning (Legendre 2008) was applied to disentangle the explanatory power of treatment from that of within - and among species variability on the respective measured parameters. The analysis was conducted using R-package *vegan* version 2.0-10. Both species and ecotypes were used as factors (their origin), with ecotypes nested in species. Variance partitioning was not possible for winter warming and frost experiment due to the presence of only one ecotype for most species.

Tukey's post hoc tests were used to distinguish significant differences among treatment levels as well as CVs of species and countries. 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 were created from map scans (Meusel & Bräutigam 1992) using ArcGIS version 10.2.2.

## **RESULTS**

Overall treatment effects on actual measured parameter values

All three extreme event simulations – spring frost, drought and warming and winter warming plus frost – affected most measured parameters related to plant performance negatively (Panel A in Fig. 3, 4 and 5). Only in the drought and warming experiment, warming did not affect biomass and had a negligible effect on C/N ratio compared to the drought treatment (Fig. 3; Table 3).

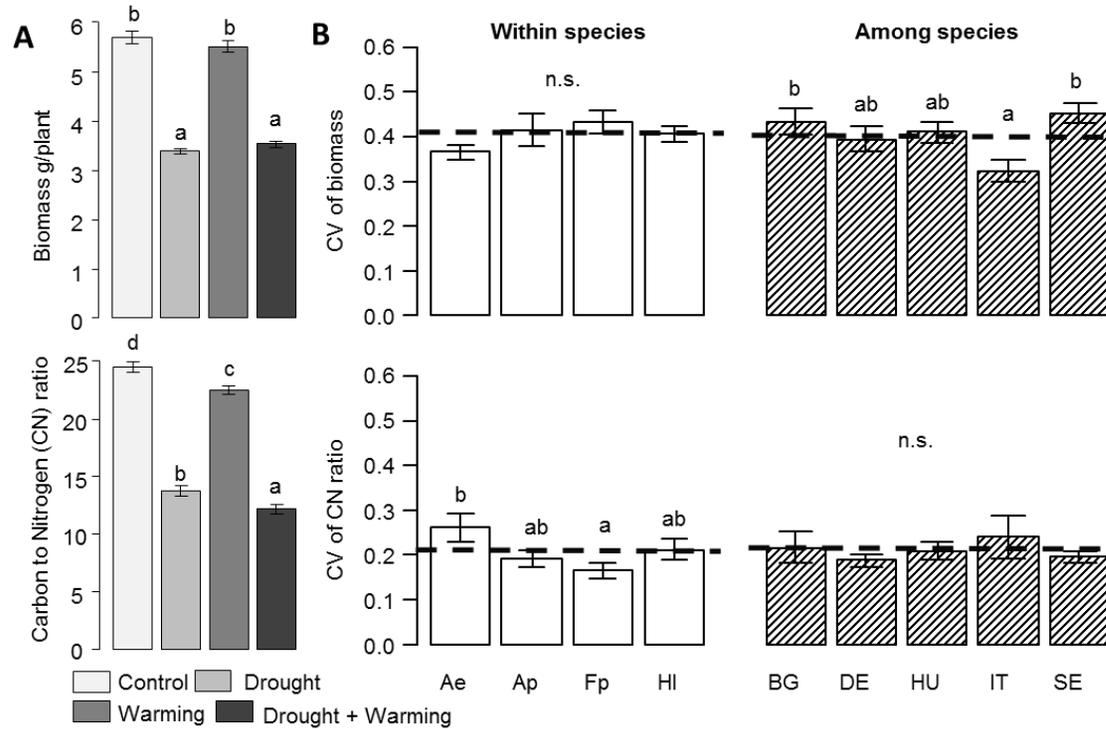
In the drought and warming experiment biomass responses were species-specific and country-specific, showing that both ecotypes and species had unique stress responses (Table 3).

In the spring frost experiment similar interactions were found for biomass and chlorophyll content parameters, although biomass responses were more-species specific (Table 3). In the winter warming plus frost experiment root  $^{15}\text{N}$  uptake and dead tissue biomass also showed species and ecotype-specific responses, with both species and ecotypes responding uniquely with respect to  $^{15}\text{N}$  uptake and species-specific accumulation of dead tissue biomass (Table 3).

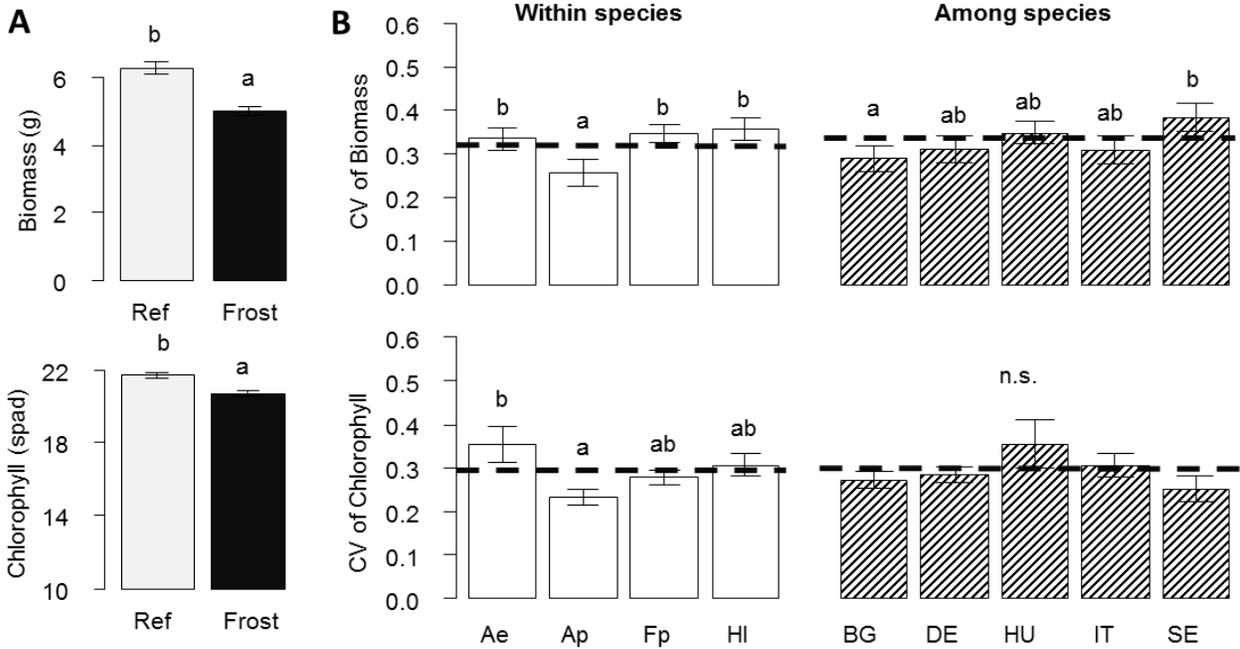
Within vs. among-species variation: differences in coefficients of variation

Variation within each species across ecotypes was compared to variation among different species in each country of seed origin, for each experiment and respective measured parameter (Table 4). Within-species variation under different extreme events (summer drought and warming 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). (Table 4: summer drought /warming experiment and spring frost experiment). 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 tissues biomass,  $^{15}\text{N}$  uptake) (Table 4: winter warming plus frost).

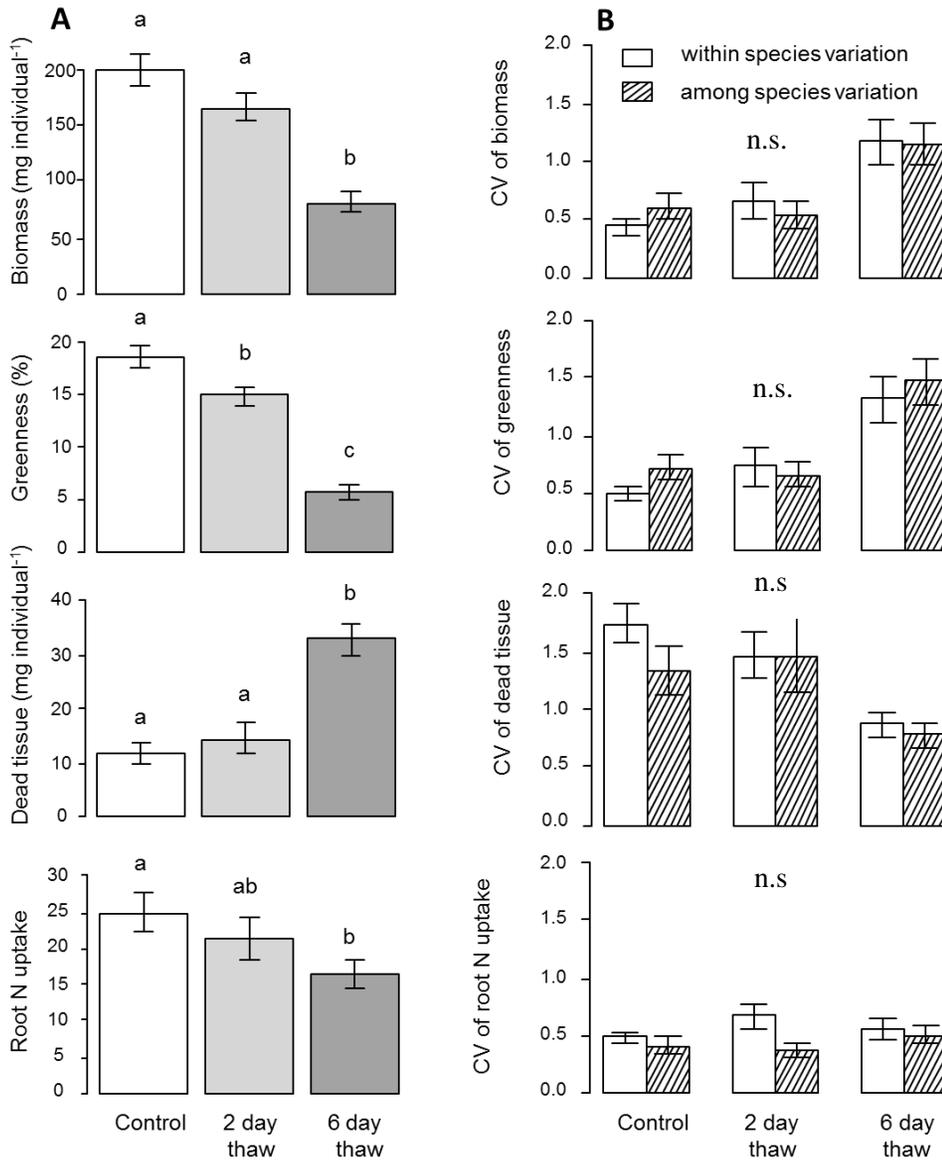
In total 77 comparisons of within- vs among-species variation were made, each one representing the within-species variation for a specific species and among-species variation in a specific location (country), for each response parameter across all treatments. In eleven of these comparisons within-species CVs significantly exceeded among-species CVs six times, being 27% higher on average (see relative effect sizes in Table 4). In the other five significantly differently comparisons, among-species CVs were on average 21% higher than within-species CVs.



**Figure 3:** Drought and warming experiment. Panel 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 / ecotypes, species and treatments are presented in Table 3 - A in Appendix. Panel 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 country (4 local ecotypes of each species) represent among-species variation (BG – Bulgaria, DE – Germany, HU – Hungary, IT – Italy, DE – Sweden) using pooled data from all treatments. Error bars denote standard errors. Different letters indicate significant treatment differences. Dashed lines indicate mean within- and among-species CVs, respectively.



**Figure 4:** Spring frost experiment. **Panel 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/ecotypes, species and treatments are presented in Table 3 - B in Appendix. **Panel 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 country (4 local ecotypes of each species) represent among-species variation (BG – Bulgaria, DE – Germany, HU – Hungary, IT – Italy, DE – Sweden) using pooled data from both reference and frost. Error bars denote standard errors. Different letters indicate significant treatment differences. Dotted lines indicate mean within- and among-species CVs, respectively.



**Figure 5:** Winter warming plus frost experiment. **Panel A:** Overall treatment effects on healthy and dead tissue biomass, greenness and root <sup>15</sup>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 Table 3 - B. **Panel 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.

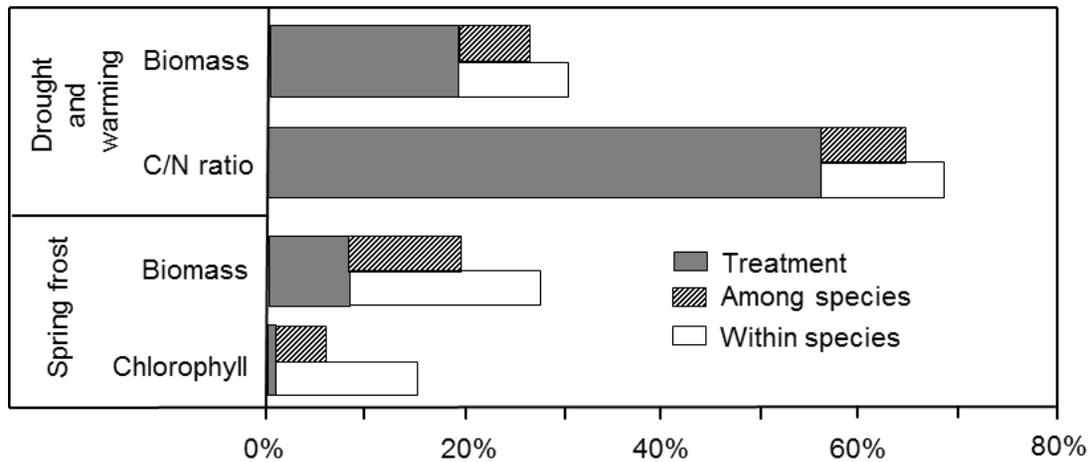
**Table 3.** Species and country specific treatment effects on each measured parameter and their interactions for drought and warming experiment (A) and for spring frost experiment (B). For the winter warming plus frost experiment (C) treatment effects on species and ecotypes of *A. elatius* as well as their interactions are presented. Significant differences are marked in bold ( $p < 0.05$ ).

Parameter	Response	F	P	
<b>A. Drought and warming experiment</b>				
Biomass (g)	Species	118.6	<.0001	
	Country	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	
	Country × Drought	3.7	<b>0.0044</b>	
	Country × Warming	0.2	0.9474	
	Species × Drought × Warming	0.9	0.4474	
	Country × Drought × Warming	0.5	0.7267	
	Biomass C/N ratio	Species	40.0	<.0001
		Country	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	
Country × Drought		2.1	0.0596	
Country × Warming		0.5	0.7838	
Species × Drought × Warming		2.5	0.0570	
Country × Drought × Warming		0.7	0.6585	
<b>B. Spring frost experiment</b>				
Biomass (g)		Species	20.8	<.0001
		Country	4.5	<b>0.0015</b>
		Spring frost	42.4	<.0001
	Species × Spring frost	3.5	<b>0.0160</b>	
	Country × Spring frost	0.6	0.6624	
Chlorophyll content (spad)	Species	59.6	<.0001	
	Country	70.4	<.0001	
	Spring frost	24.8	<.0001	
	Species × Spring frost	9.8	<.0001	
	Country × Spring frost	8.6	<.0001	
<b>C. Winter warming plus frost experiment</b>				
Biomass (g)	Species	6.6	<.0001	
	Ecotype	2.1	<b>0.0217</b>	
	Treatment	54.2	<.0001	
	Species × Treatment	1.2	0.3227	
	Ecotype × Treatment	0.1	0.40833	
Greenness (%)	Species	3.4	<b>0.0023</b>	
	Ecotype	1.9	<b>0.0416</b>	
	Treatment	67.9	<.0001	
	Species × Treatment	1.5	0.1876	
	Ecotype × 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	<b>0.0091</b>	
	Ecotype × Treatment	0.7	0.7887	
Root <sup>15</sup> N uptake (mg label per g dry weight)	Species	14.3	<.0001	
	Ecotypes	0.5	0.8177	
	Treatment	6.2	<b>0.0027</b>	
	Species × Treatment	2.2	<b>0.0236</b>	
	Ecotype × Treatment	2.3	<b>0.0169</b>	

**Table 4:** Relative effect size (% difference) for within vs. among species coefficients of variation using pooled data from all treatments for each experiment. Within-species variation in each species (4-5 ecotypes, see Table 2) is compared with among-species variation in each country (4-5 species, see table 2) for drought and warming experiment and spring frost experiment. For each within- vs. among-species comparison, the local country ecotype of each species was used as part of among-species variation and not included in within-species variation. CV = Coefficient of variation. Positive values (% difference) indicate that species variation is higher than ecotypic variation, negative values the reverse situation; NA: analysis not done due to unavailable local ecotypes for these species; 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 ( $P < 0.05$ ) are marked in bold.

CV of responses		Among species (Country)				
		Bulgaria	Germany	Hungary	Italy	Sweden
Within species						
<b>A. Drought and warming experiment</b>						
CV of biomass	<i>Arrhenatherum elatius</i>	<b>-18.7</b>	-7.8	-12.1	<b>+11.3</b>	<b>-24.0</b>
	<i>Alopecurus pratensis</i>	-4.5	+5.1	+1.3	NA	<b>-9.2</b>
	<i>Festuca pratensis</i>	-0.1	+9.1	+5.5	<b>+25.2</b>	-4.5
	<i>Holcus lanatus</i>	-6.6	+3.2	-0.7	<b>+20.3</b>	NA
CV of biomass C/N ratio	<i>Arrhenatherum elatius</i>	+17.5	<b>+28.3</b>	+20.0	+7.6	+25.5
	<i>Alopecurus pratensis</i>	-12.5	+2.2	-9.0	NA	-1.6
	<i>Festuca pratensis</i>	-29.7	-12.8	<b>-25.7</b>	-45.2	-17.1
	<i>Holcus lanatus</i>	-1.2	+12.0	+1.9	-13.3	NA
<b>B. Spring Frost experiment</b>						
CV of biomass	<i>Arrhenatherum elatius</i>	+13.5	+7.1	-3.6	+7.7	-14.6
	<i>Alopecurus pratensis</i>	-12.9	-21.3	<b>-35.2</b>	NA	<b>-49.6</b>
	<i>Festuca pratensis</i>	+16.6	+10.4	+0.1	+11.0	-10.5
	<i>Holcus lanatus</i>	<b>+19.1</b>	+13.1	+3.1	+13.6	NA
CV of chlorophyll	<i>Arrhenatherum elatius</i>	+23.1	+19.5	0.0	+13.8	+28.9
	<i>Alopecurus pratensis</i>	-6.4	-11.5	-38.3	NA	+1.6
	<i>Festuca pratensis</i>	+1.8	-3.0	-27.7	-10.1	+9.2
	<i>Holcus lanatus</i>	+11.1	+6.8	-15.6	+0.3	NA
<b>C. Winter warming and frost experiment*</b>						
CV of biomass	<i>Arrhenatherum elatius</i>	NA	-1.5	NA	NA	NA
CV of greenness	<i>Arrhenatherum elatius</i>	NA	-12.4	NA	NA	NA
CV of dead tissue	<i>Arrhenatherum elatius</i>	NA	+15.2	NA	NA	NA
CV of root <sup>15</sup> N	<i>Arrhenatherum elatius</i>	NA	+33.0	NA	NA	NA
CV of leaf <sup>15</sup> N	<i>Arrhenatherum elatius</i>	NA	+25.7	NA	NA	NA

For drought and warming and as well as spring frost experiments, partitioning of variance showed similar among- and within-species explanation of total variation, with a mean 8% of variation being explained by species (and thus ecotypes, which were nested in species), with an additional 6% of variation being explained by ecotypes alone, having accounted for species-specific differences (Fig. 6).



**Figure 6.** Variation partitioning of drought and warming as well as spring frost experiments, with respect to each measured parameter. Both species and ecotypes are factors (their origin), with ecotypes nested in species (one species has several ecotypes). Within-species differences explained an additional mean of 6 % of total variation, after accounting for treatment effects and among – species differences. No additional variation could be explained by species after accounting for the within-species factor.

## 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). Several 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, local adaptation at the continental scale results in ecotypes which react to climate extremes as differently as widely distributed common species (Figure S1) from a common origin. This strong influence of local adaptation has immediate theoretical implications for e.g. 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 genetic diversity within species may be as important in insuring ecosystem integrity in times of increasing climatic perturbation as species richness. However, 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, and as mentioned previously, assisted migration of ecotypes may be required to significantly increase the stability of an ecosystem (Kreyling *et al.* 2011). The potential for maladapted hybrids and outbreeding depression due to the mixing of ecotypes need to be evaluated despite being probably minor as compared to the potential negative effects of the assisted migration of species (Aitken & Whitlock 2013).

### Local adaptation vs. species identity

Our results demonstrate that local adaptation can match species identity in terms of influencing environmental stress responses. Interbreeding barriers, which keep co-existing species unique, can therefore match spatial or environmental separation in terms of influencing plant responses to environmental stress. The rate at which local adaptation to stress is developed, however, is largely unknown, although there are indications that it can be rapid; for example, phenotypically distinct fish ecotypes, which have much faster gene exchange rates than plants, were reduced to a single ecotype upon being placed in the same environment and allowed to mate for 12 generations (Moran & Alexander 2014). The speed of evolution of local adaptation should therefore 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) 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 only in 71% of transplant studies (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 at locations as cold as Abisko, northern Sweden (Bokhorst *et al.* 2008). Field experiments have shown comparable growth reduction with extreme winter warming to that

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 to winter climate change was realistic.

## CONCLUSIONS

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 that among-species trait variation dominates under non-stressful conditions. 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.

We see five important research questions arising from our findings: (1) What is the speed of evolution of local adaptations and plant ecotypes? (2) What happens if geographically isolated ecotypes are mixed by humans with respect to performance and response variability (Fig. 1)? (3) How does the within-species variability in stress responses vary among species and what drives this variability (species, functional groups, generation length, range size etc.)? (4) What causes 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?

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# Manuscript 5: Nitrogen leaching is enhanced after a winter warm spell and controlled by plant community composition in temperate zone mesocosms

To be submitted

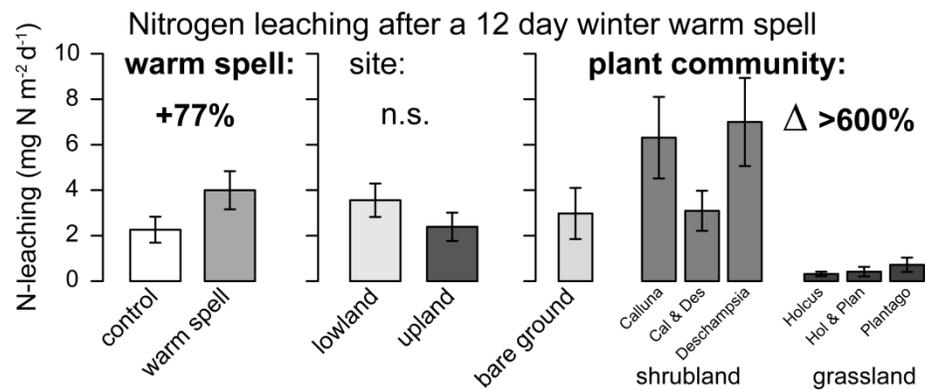
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## Graphical Abstract:



## Highlights:

- A winter warm spell induced 77% increased N-leaching in temperate mesocosms
- Plant community composition and performance controlled N-leaching
- Leaching was >600% higher in shrubland than in grassland and exceeded bare ground
- A colder and wetter site showed higher N-leaching
- Responsiveness to the winter warm spell did not differ among a cold and a warm site

## **Abstract**

Leaching of nitrogen (N) from ecosystems, commonly thought to be caused by excessive atmospheric N deposition or fertilization, poses serious environmental problems. Extreme events such as winter warm spells, however, could also exacerbate N-leaching by disrupting biogeochemical cycles and will become more frequent with climate change. Here, we used a mesocosm/ lysimeter approach to investigate N-leaching in response to a 12-day winter warm spell at two field sites with contrasting winter climate and in seven different temperate plant communities (n = 140 lysimeters). Overall, 2.2 mg N l<sup>-1</sup> leaching was observed, summing up to a mean of 1.26 kg N ha<sup>-1</sup> over the 49 days of observations in late winter/ early spring. The extreme winter warm spell resulted in 77% increase of N-leaching after the warm spell (up to 18 mg N l<sup>-1</sup>). This leaching was affected by the climatic setting with stronger leaching under colder ambient conditions. The difference between the sites can be explained by plants becoming photosynthetically activated by the warm spell, then being frost-damaged at the cold site due to missing insulation by snow cover, resulting in reduced N uptake by the plants. N-leaching furthermore differed by >600% among contrasting plant communities with almost no leaching from grassland communities and strongest leaching from shrubland communities that even surpassed leaching from bare ground controls. We conclude that winter warm spells can affect the biogeochemistry of temperate ecosystems with plant performance and plant community composition controlling the amount of N leaching.

## **Keywords:**

winter ecology, freezing-thawing, frost, warming pulse, heat wave, nitrogen loss, nutrient leaching

## **1. Introduction:**

Leaching of nitrogen from ecosystems is a long-standing environmental problem causing numerous detrimental effects including soil and surface water acidification, leaching of soil minerals, eutrophication and hypoxia of inland and coastal waters, or the pollution of ground and drinking water (Vitousek et al., 1997; Zhang et al., 1996; Syswerda et al., 2012). Nitrogen leaching is commonly linked to fertilization and atmospheric nitrogen deposition, i.e. excessive nitrogen input into ecosystems which otherwise show tight nitrogen cycling (Aber et al., 1993; Dise and Wright, 1995; Dise et al., 2009). Low soil pH and C:N ratios further exacerbate nitrogen losses from ecosystems (Dise and Wright, 1995).

Climatic parameters such as mean temperature and precipitation also affect nitrogen leaching from ecosystems with generally increased leaching under cooler and wetter conditions (Dise et al., 2009; Patil et al., 2010; Zhao et al., 2010). With ongoing climate change generally enhancing warmer and dryer conditions (IPCC, 2013), reduced nitrogen leaching is to be expected. However, contradicting results have emerged from studies quantifying nitrogen leaching under climate change scenarios in temperate or colder ecosystems and winter emerged to be a crucial season to consider for biogeochemistry and vegetation performance (Campbell et al., 2005; Kreyling, 2010). There, climate warming effects are modified by reductions in the insulating snow cover which can lead to “colder soils in a warmer world” (Groffman et al., 2001a). The resulting soil frost is known to trigger nitrogen leaching (Fitzhugh et al., 2001 & 2003; Groffman et al., 2001b; Joseph and Henry, 2008; Campbell et al., 2014). Chronic winter warming in a temperate old field, however, had no effects on nitrogen leaching (Turner and Henry, 2010). Other studies, report increased nitrogen leaching due to generally warmer winters and no effects of increased soil frost on nitrogen leaching (Kaste et al., 2008). A reduced snow cover can further result in both, decreased and increased nitrogen leaching, potentially depending on complex interactions among nitrogen deposition, soil temperature, and soil water flow (de Wit et al., 2008; Stuanes et al., 2008).

Vegetation type and plant performance are also important drivers of nitrogen leaching (Hooper and Vitousek, 1998; Knops et al., 2002). Vegetation response to warming and frost might therefore - at least in part - explain site-specific post-frost N-leaching. Increased

mineralization in warm winters can be effectively immobilized by early plant growth (Patil et al., 2010; Shibata et al., 2013). N-leaching, however, can occur if plant uptake is reduced due to frost damage to the plant roots (Groffman et al., 2001b; Campbell et al., 2014). Likewise, Matzner and Borken (2008) conclude that the increased nitrogen leaching in response to repeated freeze-thaw cycles observed in several studies might rather be due to reduced plant uptake than increased nitrogen mobilization. Plant responses to winter climate change might thus be crucial for nitrogen cycling. Reduced snow cover due to winter warm spells followed by hard frost, for instance, can substantially damage subarctic vegetation (Bokhorst et al., 2009). Similar effects are also described for some temperate plant species (Schuerings et al., 2014). In response to recurrent soil freeze-thaw cycles, strong differences between species are described, with certain grasses being more opportunistic to nitrogen availability in winter (Kreyling et al., 2008) than dwarf shrubs (Kreyling et al., 2010), potentially fostering nitrogen leaching.

Contrasting to colder sites (Groffman et al., 2001a), at warmer temperate sites the occurrence of soil frost decreases with global warming despite the loss of snow cover (Kreyling and Henry, 2011). Yet soil temperature can still be expected to become more variable under future climatic conditions, with unclear effects on ecological and biogeochemical processes (Kreyling, 2010). Here, plants might be able to profit from warmer winters and increased nitrogen mineralization by improved and earlier growth. Roots penetrating deeper into the soil after winter warming, however, can also be interpreted as signs for nitrogen leaching even in the absence of soil frost (Schuerings et al., 2013).

Taken together, there is evidence that climate change, in particular increased winter temperature variability, can result in nitrogen leaching. Nonetheless, findings from different geographical locations and different vegetation types are controversial. Consequently, we have designed an experiment testing the influence of warmer and more variable winter temperatures (one winter warm spell of 12 days) on ecosystems differing in climatic settings and vegetation types or species identities. Nitrogen leaching together with various indicators of plant performance (photosynthetic activity, root length, greenness and biomass production) was quantified during and/or after the winter warm spell. We hypothesized that (1) a prolonged winter warm spell results in increased nitrogen leaching. We further expected that (2) plant performance drives nitrogen leaching due to species- and site-specific effects with imprints of the warm spell

carrying over into the growing season. Specifically, we expected more severe nitrogen leaching if plants acquired more frost damage after being deacclimated by the warm spell (i.e. at the colder site) and in response to less opportunistic vegetation (shrubland as compared to grassland).

## 2. Material & Methods

### 2.1. Experimental design and site description

We tested the effects of a prolonged winter warm spell on nitrogen leaching in temperate shrubland and grassland communities in a lysimeter experiment at two sites with contrasting winter conditions. The lowland site was located in the Ecological-Botanical Garden of the University of Bayreuth (49° 55' 36.32", 11° 34' 57.28", 358 m asl) and the upland site was located at the Waldstein mountain in the Fichtelgebirge (50° 8' 35.81", 11° 51' 50.92", 781 m asl.). See Table 1 for a comparison of climatic conditions at both sites.

**Table 1:** Comparison of the climate at both experimental sites. Period of record 1998-2013 (except for soil frost: 2002-2013). Data: courtesy by T. Foken, Micrometeorology, University of Bayreuth.

Climate parameter	Lowland	Upland
Mean annual temperature (°C)	8.7	6.4
Mean winter temperature (DJF, °C)	0.1	-2.1
Mean annual precipitation (mm)	719	983
Mean winter precipitation (DJF, mm)	154	239
Soil frost (days with daily temperature < 0°C at -5 cm)	13	43

The experiment consisted of three fully crossed factors: (1) application of winter warming pulses versus ambient control conditions, (2) two experimental sites with different winter climate and (3) six different plant communities and bare ground conditions. Three grassland communities (monocultures of *Holcus lanatus* and *Plantago lanceolata*, and a community with a mix of both species) and three shrubland communities (monocultures of *Calluna vulgaris* and *Deschampsia flexuosa* and a community with a mix of both species) were planted. Plant communities were blocked and randomly assigned to the warming-pulses manipulation and ambient control.

Temperature manipulation blocks, and, hence, each factorial combination, were replicated five times. This setup was fully replicated at both experimental sites. For the 140 plots, plastic barrels with 0.2 m<sup>2</sup> surface (50 cm diameter) and 80 cm depth were used as mesocosms. Each of six mesocosms per treatment was placed in each corner of a hexagon with a distance of 30 cm between mesocosms and at least 50 cm separation from the hexagon edge. One mesocosm with bare ground conditions was placed in the center of each hexagon. All space between the mesocosms was filled with the same substrate as used within the mesocosms. The soil substrate was homogenized loamy sand (77 % sand, 16 % silt, 7 % clay) from a nearby sand quarry with a pH=7.35 (measured in 1 M KCl), a total carbon content of 2.37 % and a total N content of 0.11 %. The barrels were attached with outlet hoses at the bottom of each mesocosm, ensuring that the mesocosms functioned as zero tension lysimeters. Sixteen plants per plot were planted in a systematic grid in May 2010. All plants were grown from seeds in January 2010 except for the dwarf-shrub *C. vulgaris* which was obtained as 2-year old individuals in February 2010. All species present in this experiment are very common perennial species in Central Europe.

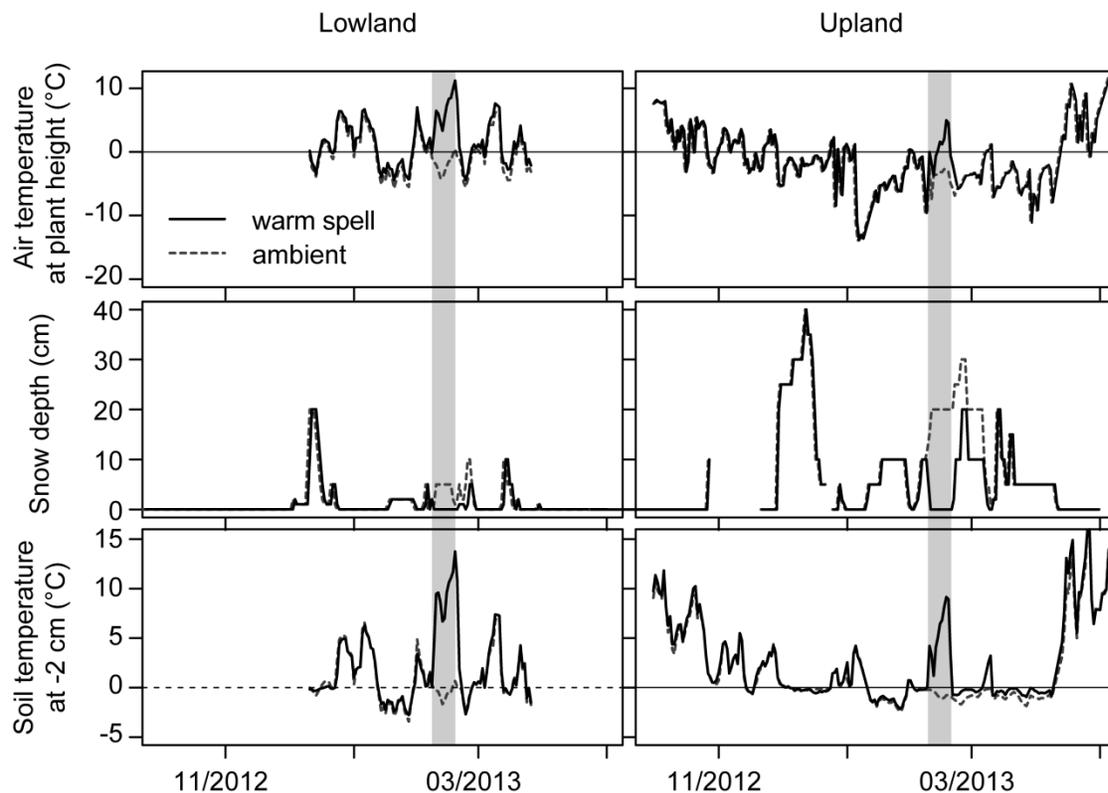
## 2.2. Warm spell manipulation

One winter warm spell was applied for 12 days in winter 2012/13 with six IR-heating lamps (IOT/90, 250 W, Elstein, Germany) located in between the mesocosms at a height of 60 cm and surface heating wires (deviflex DTIP, DEVI, Vejle, Denmark; distance 20 cm, 400 W per block), which resulted in 1900 W per block (7 mesocosms). Wind speed and snow drift were minimized by wind nets up to 1 m height around all blocks (treatment and control) during the warming phase. The ambient control plots were equipped with dummy lamps. The warm spell started at the lowland site at February 7<sup>th</sup> and lasted until February 18<sup>th</sup>. At the upland site, the warm spell was shifted by one day in order to allow for sampling at equal time intervals after start of warming (February 8<sup>th</sup> - 19<sup>th</sup>). Due to electronic malfunctioning, two warming blocks at the upland site were continuously warmed throughout the winter and therefore excluded from the analyses.

Soil (-2 cm; once in every treatment block) and air temperature (+5 cm; one per treatment and experimental site) were measured hourly by thermistors (B57863-S302-F40, EPCOS) connected

to a datalogger (dl2, Delta). Snow height was measured each morning via webcam pictures at measuring sticks.

The winter warm spell manipulation successfully decreased snow cover and warmed air and soil temperatures (Figure 1). Manipulation effects on air temperatures increased steadily over the duration of the warm spell, reaching maximum daily mean temperatures at the lowland site of 11.2°C as compared to 0.5°C in the control, and 5.0°C versus -2.7°C, respectively, at the upland site. Soil temperatures at -2 cm showed even stronger manipulation effects in maximum daily temperatures with 13.7°C vs. 0.7°C at the lowland site, and 9.1°C vs. -0.2°C at the upland site, respectively. Despite missing snow cover at the end of the warm spell in the manipulation blocks, neither air nor soil temperature of the manipulated plots dropped below ambient control conditions. The warming treatment therefore did not result in colder soils after the warming compared to the controls. Nevertheless, moderate air and soil frost occurred after the end of the warm spell manipulation at both sites and in both treatments.



**Figure 1:** Air temperature, snow depth and soil temperature at both experimental sites over winter 2012/13. Displayed are daily mean values for the warm spell manipulation and ambient control.

### 2.3 Response parameters

Leachate from the zero-tension lysimeters was collected in belowground, well-insulated canisters. All canisters were emptied and cleaned at the onset of the warm spell. Volume of leachate and representative samples were collected at the last day of the warm spell manipulations in all 140 plots. A second sampling took place on March 27<sup>th</sup> (lowland) and 28<sup>th</sup> (upland), i.e. 37 days after the first sampling. Samples were kept frozen at -30°C before filtration (Typ 15 A Blauband; Roth, Karlsruhe, Germany) and quantification of nitrate and ammonium concentrations by flow injection analysis (BayCEER Analytical Chemistry, Bayreuth, device: FIA-LAB, MLE GmbH, Dresden, Germany). Below, we report the sum of nitrate and ammonium, with nitrate dominating the signal (overall: 98.6% nitrate, 1.4% ammonium).

Effective quantum yield of the photosystem II ( $\Delta F/F_m'$ ) of the plants during the winter warm spell was quantified in *P. lanceolata* by a MINI-PAM-Fluorometer (Heinz Walz GmbH, Effeltrich, Deutschland) in light acclimated leaves, as  $\Delta F/F_m' = (F_m' - F_t)/F_m'$  where  $F_t$  and  $F_m'$  are the actual and maximal chlorophyll fluorescence under ambient conditions, respectively (Genty et al., 1989).  $\Delta F/F_m'$  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-Nordenkamp et al., 1989; Werner et al., 2002). Leaves were measured in situ in the natural position with a leaf clip holder after allowing  $F_t$  to stabilize (about 20 seconds). 5-10 leaves were measured per plot and sampling date (6<sup>th</sup> and 12<sup>th</sup> day of the warm spell).  $\Delta F/F_m'$  is dependent on the incident photosynthetic active radiation (PAR). Measurements were therefore restricted to similar PAR-conditions ( $20 \mu\text{mol m}^{-2} \text{s}^{-1} < \text{PAR} < 130 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Care was taken to discharge data out of measurements range (i.e.  $F_m' > 2000$  and/or  $\Delta F_t - F_m' < 50$ ). Snow was brushed off from plants prior to measurements and then carefully re-deposited.

Plant activity early in the growing season was quantified by digital pictures, taken under standardized light conditions early in April. For this purpose, a portable light-tight box of 60 cm in diameter with a camera (Nikon D2x) and artificial lighting was used. The calculation of the greenness was done by converting the RGB-photos into black and white images in Adobe Photoshop (version 6). Black pixel percentage was then processed in R with the help of the additional packages `rgdal` version 0.8-16 and `raster` version 2.2-32

Root length was determined via a minirhizotron technique in a clear plastic tube (5 cm diameter) which was installed 45 cm deep at a 45° angle in each mesocosm. The above-ground part of the tube was covered with adhesive aluminium foil and closed with a rubber cap to prevent entry of light and temperature shifts. Pictures were taken with a root scanner at the start of the warm spell and again in May 28<sup>th</sup> (lowland) and June 03<sup>rd</sup> (upland). The root scanner was built from an ordinary computer scanner (Optic slim 2400+) mounted on a metal pole that was turned by an electric motor. The scanner took pictures (18 cm x 21.6 cm depth) of an angle of around 300°. Root turnover was calculated as the sum of differences in root length (roots lost + new roots) between the sampling times (quantified using the software Rootfly, Birchfield & Wells 2007, Version 2.0.1) for each tube in 4 cm by 7 cm details starting at the soil surface and looking directly upwards.

Total above-ground biomass was harvested destructively on May 28<sup>th</sup> (lowland) and June 03<sup>rd</sup> (upland). Grassland plots were cut to a height of 3 cm twice per year before the treatment, resembling local agricultural routines in these semi-natural ecosystems. Shrubland plots had not been harvested before. For both vegetation types, all standing biomass was sampled in paper bags, dried to constant weight for 48 hours at 75°C, and weighed.

## 2.4 Data analysis

Linear mixed-effect models combined with analysis of variance (ANOVA) were applied to test for significant treatment effects. Block identity was set as a random effect, thereby accounting for the blocked design. We tested for the fixed factors warm spell treatment, site, plant community, and date (the latter only for  $\Delta F/Fm'$ ) during and after the warm spell manipulation. All possible interactions of site and plant community with the warm spell treatment were included as fixed effects (s. Table 2 for all tested interactions). For the analyses of  $\Delta F/Fm'$ , plot identity and PAR were used as additional random effects, thereby accounting for the nested sampling within the plots and the dependence of  $\Delta F/Fm'$  on PAR. In addition, community was not tested as main affect but accounted for as random affect because the target plant *P. lanceolata* only occurred in two of the seven communities. Significant interactions with the warming pulses treatment were tested by Tukey post hoc comparisons. Before statistical analysis, we checked normality and homogeneity of variance by examining the residuals versus fitted plots and the

normal qq-plots of the linear models (Faraway, 2005). Square root transformation was applied to nitrogen leaching data, rank transformation to  $\Delta F/F_m'$  data, and square root transformation to biomass and greenness data in order to improve these model characteristics. Alpha was set to  $p < 0.05$ . All statistical analyses were performed using R 3.0.2 (R Development Core Team 2013) and the 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.

### 3. Results:

Considerable N-leaching in winter and early spring was observed with an overall mean of 2.2 mg N l<sup>-1</sup> leachate equaling a mean of 1.26 kg N ha<sup>-1</sup> over the 49 days of the observation period. N-leaching, however, was highly variable over time and among treatments with maxima reaching 18.3 mg N l<sup>-1</sup>. The warm spell did not induce increased N-leaching during the warming phase (Table 2), yet N-leaching per day after the warm spell increased by 77% in comparison to control conditions (Figure 2, Table 3), reaching means of 2.6 mg N l<sup>-1</sup> in the warm spell manipulation versus 1.9 mg l<sup>-1</sup> in the ambient control or 1.08 kg N ha<sup>-1</sup> in the warm spell manipulation versus 0.61 kg N ha<sup>-1</sup> in the control over the 37 days observation period in early spring.

N-leaching during the warm spell in winter was affected by site climate with N-leaching being 69% higher at the colder and more snow-rich upland site (Figure 2, Table 2). This difference ceased in spring when no significant difference among the sites was observed any longer (Table 3).

Plant community composition strongly affected N-leaching both during the warming manipulation period in winter and in early spring (Table 2 and 3). Generally, N-leaching from the dwarf-shrub communities was 669% higher than from the grassland communities and this pattern hold true for both time periods (Figure 2). In spring, N-leaching from the dwarf-shrub monocultures even surpassed leaching from bare ground controls. The plant community was by far the most important explanatory variable for N-leaching in spring (Table 3).

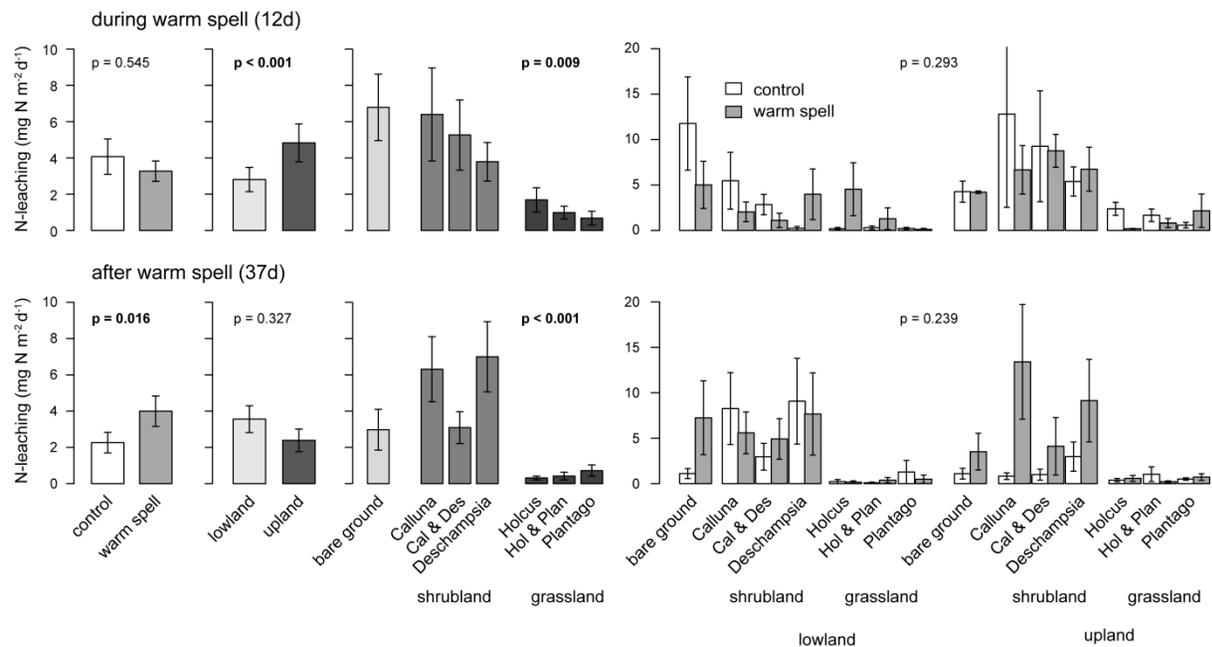
**Table 2:** ANOVA results of the linear mixed effect models for the parameters measured during the 12 d warm spell in February. Block ID was used as random effect in all models.

	<b>N-leaching</b>		<b><math>\Delta F/F_m'</math> of <i>P. lanceolata</i></b>	
	F	p	F	p
Warm spell	0.4	0.545	<b>129.9</b>	<b>&lt;0.001</b>
Site	<b>19.5</b>	<b>&lt;0.001</b>	<b>172.5</b>	<b>&lt;0.001</b>
Community	<b>3.1</b>	<b>0.009</b>		
Warm spell : Site	0.1	0.724	0.4	0.535
Warm spell : Community	0.4	0.867		
Warm spell : Site : Community	1.2	0.293		

**Table 3:** ANOVA results of the linear mixed effect models for the parameters measured in spring after the warm spell in February. Block ID was used as random effect in all models.

	<b>N-leaching</b>		<b>Above-ground biomass</b>		<b>Greenness</b>		<b>Root turnover</b>	
	F	p	F	p	F	p	F	P
Warm spell	<b>6.0</b>	<b>0.016</b>	0.3	0.597	<b>7.3</b>	<b>0.009</b>	0.1	0.733
Site	1.0	0.327	3.8	0.053	<b>26.0</b>	<b>&lt;0.001</b>	<b>4.7</b>	<b>0.036</b>
Community	<b>9.2</b>	<b>&lt;0.001</b>	<b>6.9</b>	<b>&lt;0.001</b>	<b>4.5</b>	<b>0.003</b>	0.5	0.757
Warm spell : Site	0.9	0.341	0.2	0.678	1.2	0.284	0.2	0.680
Warm spell : Community	0.9	0.514	1.1	0.35	1.7	0.159	0.3	0.884
Warm spell : Site : Community	1.3	0.239	<b>5.9</b>	<b>&lt;0.001</b>	<b>2.2</b>	<b>0.039</b>	*	

\* not available due to missing data

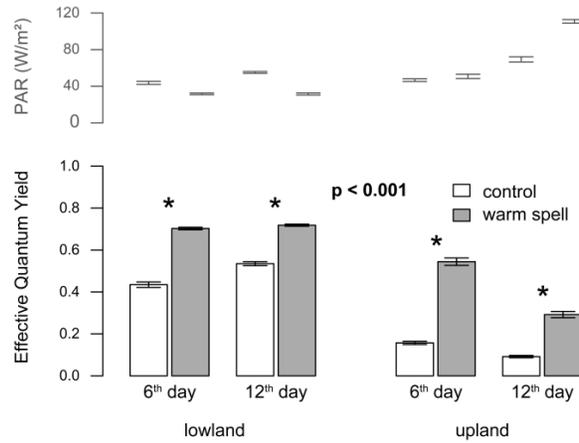


**Figure 2:** N-leaching (in  $\text{mg N m}^{-2} \text{d}^{-1}$ ) during the 12 d warm spell in February (upper panel) and after the warm spell (37 d from February to April; lower panel). Shown are mean values and standard errors over  $n=140$  for each single main factor and their threefold interaction. P-values according to ANOVA are given for each factor, full ANOVA results are shown in Tables 2 and 3.

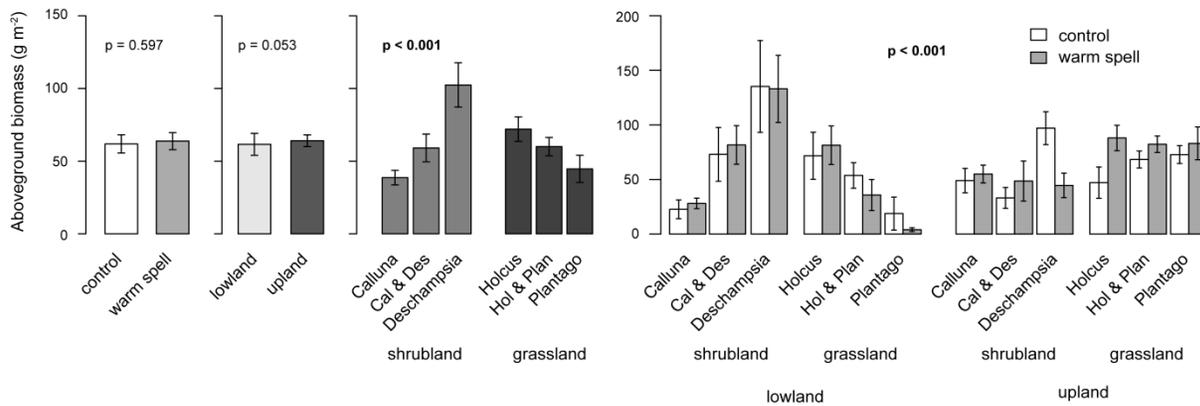
Within the first six days of the winter warm spell, the plants showed a clear activation of photosynthetic activity as indicated by the increased  $\Delta F/F_m'$  in the warm spell manipulation as compared to control conditions (Figure 3). The temporal responses of  $\Delta F/F_m'$  to the warm spell differed among the two sites (interaction among warm spell treatment, time, and site:  $p < 0.001$ ): at the lowland site,  $\Delta F/F_m'$  reached a high constant level 6 and 12 days into the warm spell (0.70 and 0.72 respectively). At the upland site the  $\Delta F/F_m'$  increase due to the warm spell was considerably higher (relative to the control) than at the lowland site at day 6, though the absolute values remained slightly lower (0.54 at day 6). Moreover  $\Delta F/F_m'$  decreased at day 12 to 0.29. This decrease over time at the upland site might indicate frost damage to the activated tissue.

Aboveground biomass in April after the warm spell manipulation showed no significant treatment effect (Figure 4). Single species, however, showed varied responses to the warm spell (ANOVA threefold interaction warm spell manipulation, site, species composition:  $p < 0.001$ ). Species specific differences in the response to the warm spell occurred mainly at the upland site where biomass of *Deschampsia flexuosa* was strongly reduced in comparison to control

conditions while *Holcus lanatus* biomass even increased in comparison to control conditions. The other species, however, remained largely unaffected by the warm spell manipulation.

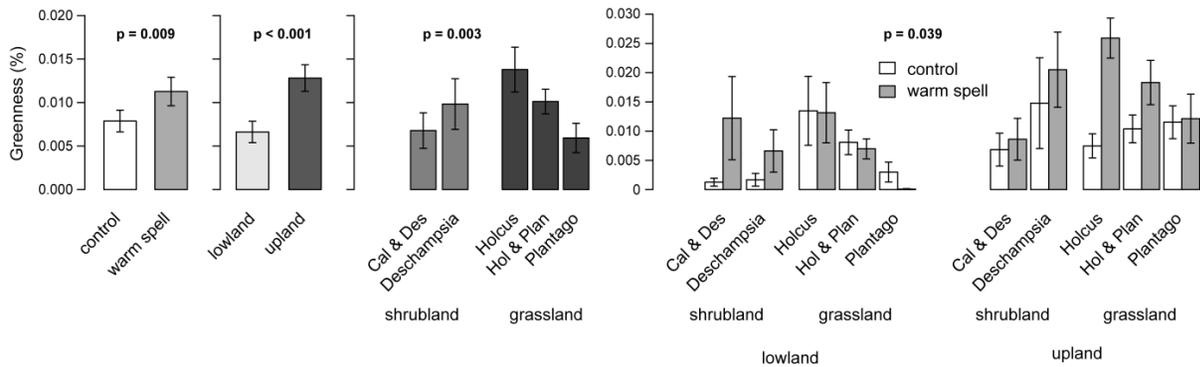


**Figure 3:** Effective Quantum yield ( $\Delta F/F_m'$ ) reflecting the photosynthetic activation due to the warm spell manipulation (grey bars) in comparison to control conditions (white bars) 6 and 12 days into the warm spell manipulation period. Shown are mean values and standard errors. P-value of the threefold interaction among warm spell manipulation, site, and time according to ANOVA are given. For comparison, mean photosynthetically active radiation for the measurements are provided in the upper panel. In the linear mixed effects model PAR was inserted as a random effect in order to account for differences in PAR among the main effect groups.



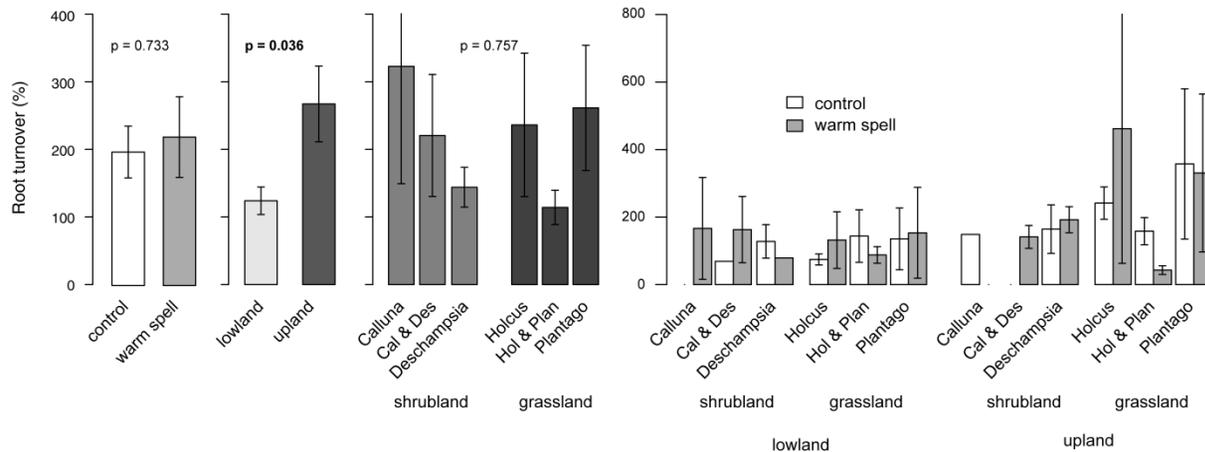
**Figure 4:** Aboveground biomass per mesocosm in spring after the winter warm spell manipulation. Shown are mean values and standard errors over  $n=120$  for each single main effect and their threefold interaction. P-values according to ANOVA are given for each factor, full ANOVA results are shown in Table 3.

Greenness in April was increased in the warm spell manipulation as compared to control conditions and greenness was higher at the upland site (Figure 5). Again, single species showed varied responses to the warm spell (ANOVA threefold interaction warm spell manipulation, site, species composition:  $p = 0.039$ ). Species specific differences in the response to the warm spell occurred mainly at the upland site where a strong increase in greenness of *Holcus lanatus* in the warm spell manipulation as compared to control conditions was observed. No species, however, showed a negative warm spell manipulation effect in greenness in spring.



**Figure 5:** Greenness per mesocosm in spring after the winter warm spell manipulation. Shown are mean values and standard errors over  $n=120$  for each single main effect and their threefold interaction. P-values according to ANOVA are given for each factor, full ANOVA results are shown in Table 3. Note that data for *Calluna* in monoculture is missing.

Root turnover from winter to spring showed large random variation within treatment groups (Figure 6). The only factor yielding significant differences in the ANOVA ( $p = 0.036$ ) was the main site effect with more than twice the turnover rates at the upland site as compared to the lowland site.



**Figure 6:** Root turnover from winter to spring. Shown are mean values and standard errors over  $n=88$  for each single main effect and their threefold interaction. No factor interaction yielded statistical significance, threefold interaction not testable due to missing values. P-values according to ANOVA are given for each factor, full ANOVA results are shown in Table 3.

#### 4. Discussion:

Considerable amounts of nitrogen leached out of the studied mesocosms (on average  $2.2 \text{ mg N l}^{-1}$  leachate or  $1.26 \text{ kg N ha}^{-1}$  over the 49 days of observation) despite medium atmospheric input ( $12 \text{ kg ha}^{-1} \text{ a}^{-1}$ , Matzner et al., 2004) and relatively high pH (7.3) of the soil substrate. Generally, no significant N-leaching is reported to occur below a deposition threshold of about  $10 \text{ kg ha}^{-1} \text{ a}^{-1}$  and observations imply only occasional leaching at atmospheric depositions of  $10\text{-}25 \text{ kg ha}^{-1} \text{ a}^{-1}$ , mainly from sites with low pH (Dise and Wright, 1995). The 12d winter warm spell induced a 77% increase in N-leaching during the 37 days from the end of the warm spell manipulation to spring. Winter warm spells as another source of N-leaching is a novel finding. Up until now, winter N-leaching was mainly attributed to soil freezing (Fitzhugh et al., 2001; Joseph and Henry, 2008; Matzner and Borken, 2008) and not to winter warming (Turner and Henry, 2010). Less snow and higher soil temperature variability during the winter is further related to lower N levels in the soil solution, indicating less microbial activity under these conditions (Durán et al., 2014). In line with this, increased N-leaching after frost events in winter has been linked to reduced uptake by the vegetation due to frost damage rather than increased N mobilization (Groffman et al., 2001b; Matzner and Borken, 2008; Campbell et al., 2014). The explanation for the observed increase of N-leaching after the prolonged warm spell in our study, however, might be similar: The plants showed clear signs of increased photosynthetic activity

due to the warm spell manipulation (Figure 3), which is only possible at the expense of reduced frost tolerance as cryoprotective carbohydrates are respired. This may potentially lead to frost damage right after the warm spell manipulation, or, as indicated by the reduced  $\Delta F/F_m'$  at the upland site towards the end of the warm spell, already during cold nights during the warm spell. Similar patterns are reported for natural (Bokhorst et al., 2008) and experimental warm spells (Bokhorst et al., 2009). Deacclimation of the plants within days (Kalberer et al., 2006) may therefore have induced frost damage despite minimum temperatures not being different among the warm spell and the control treatments. Absolute minimum temperatures have therefore to be viewed relative to the plant cold acclimation stage. Root damage due to freezing has already been shown to be linked to N-leaching (Tierney et al., 2001; Cleavitt et al., 2008). The high variation in root turnover in our study, however, does not allow for a sound identification of its role on N-leaching, even if the higher root turnover at the upland site fits well with the observed patterns of N-leaching.

Plant community composition was the factor contributing strongest to N-leaching with leaching differing by several hundred percent among vegetation types both during and after the warm spell manipulation. The decisive role of vegetation type and plant performance for N-leaching is well known (Hooper and Vitousek, 1998; Knops et al., 2002). However, our data furthermore indicates that the responsiveness of the plants themselves to the warm spell were species-specific (biomass and greenness). Some species, here the species representing the shrubland, allowed rates of N-leaching which even surpassed the bare ground controls. Changes in species compositions due to specific responsiveness to altered winter conditions might therefore affect N-leaching, as already suggested by Schuerings et al. (2013 & 2014). As an emerging general pattern, we can support the hypothesis that grasses are more responsive to winter climate perturbations than dwarf shrubs (Kreyling et al., 2010). In any case, we can conclude that plant species composition and plant performance plays a crucial role in winter N-dynamics.

Our data further supports earlier findings of increased N-leaching under generally cooler and wetter conditions (Dise et al., 2009; Patil et al., 2010; Zhao et al., 2010). The effects of the winter warm spell on N-leaching, however, were indifferent among our two study sites with contrasting winter climates (no significant interaction among site and warm spell manipulation). This implies

some generality of the observed N-leaching after winter warm spells across contrasting winter climates. A reduced snow cover, which is quite probable with ongoing climate change (Kreyling and Henry, 2011), is reported to potentially result in both, decreased and increased N leaching, depending on complex interactions among nitrogen deposition, soil temperature, and soil water flow (Wit et al., 2008; Stuanes et al., 2008). Based on our findings, these contrasting results may further be explainable by plant community composition and plant performance.

## **5. Conclusions**

We report considerable N-leaching from well-established mesocosms despite only medium input ( $\sim 12 \text{ kg ha}^{-1} \text{ a}^{-1}$ ) and high pH (7.3) of the soil substrate. A winter warm spell of 12 d induced a 77% increase in N-leaching. Photosynthetic activation of the plants during the warm spell might have led to increased frost damage after the warm spell, thereby explaining the increase in N-leaching by reduced plant uptake. The colder and wetter site in our study showed higher N-leaching, the responsiveness to the winter warm spell, however, did not vary among the two study sites. Our data strongly emphasize the decisive role of the plant community composition for N-leaching as N-leaching was several hundred percent higher in shrubland communities than in grassland communities and even surpassing bare ground controls in the former. Community composition itself might change in face of more variable winter temperatures as the response of the plants to the warm spell manipulation was strongly species-specific. Thereby, winter warm spells might cause lasting effects on plant communities and, consequently, in N-leaching patterns.

## **Acknowledgements:**

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## **Manuscript 6: Increased winter soil temperature variability enhances nitrogen cycling and soil biotic activity in temperate heathland and grassland mesocosms**

### **Journal (Biogeosciences) Published**

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### **ABSTRACT**

Winter air temperatures are projected to increase in the temperate zone, whereas snow cover is projected to decrease, leading to increased soil temperature variability, and potentially to changes in nutrient cycling. Here, we experimentally evaluated the effects of increased winter soil temperature variability on selected aspects of the N-cycle in mesocosms containing different plant community compositions. The experiment was replicated at two sites, a colder mountainous upland site with high snow accumulation and a warmer and dryer lowland site.

Increased soil temperature variability enhanced soil biotic activity for both sites during winter, as indicated by 35% higher nitrogen (N) availability in the soil solution, 40% higher belowground decomposition and a 25% increase in the potential activity of the enzyme cellobiohydrolase. The mobilization of N differed between sites, and the <sup>15</sup>N signal in leaves was reduced by 31% in response to winter warming pulses, but only at the cold site, with significant reductions occurring for three of four tested plant species at this site. Furthermore, there was a trend of increased N leaching in response to the recurrent winter warming pulses.

Overall, projected winter climate change in the temperate zone, with less snow and more variable soil temperatures, appears important for shifts in ecosystem functioning (i.e. nutrient cycling). While the effects of warming pulses on plant N mobilization did not differ among sites, reduced plant <sup>15</sup>N incorporation at the colder temperate site suggests that frost damage may reduce plant N uptake in a warmer world, with important implications for nitrogen cycling and nitrogen losses from ecosystems.

## 1 Introduction

Winter soil temperature is an important driver for many ecological and biogeochemical processes in the cold-temperate and boreal zone, and it can influence the activity of plants and soil biota (Matzner and Borken, 2008; Kreyling, 2010). While microbial activity and nitrogen (N) cycling continue below freezing (Clein and Schimel, 1995; Mikan et al., 2002), higher mean soil temperatures are generally expected to cause exponentially higher soil biotic activity (Rustad et al., 2001; Melillo et al., 2002). Consequently, winter warming can result in increased N mineralization and N availability in the soil solution in the following growing season (Turner and Henry, 2010). Warmer soils over winter increase soil biotic activity, e.g. soil respiration, decomposition by soil fauna and microbes, higher enzymatic activity, higher N mineralization, etc. This holds true especially towards the end of winter, and can accelerate plant productivity (Schuerings et al., 2013). Since plants are capable of winter N uptake (Grogan et al., 2004; Andresen and Michelsen, 2005), their activity could counteract N leaching (Patil et al., 2010). The general effectiveness of plants in taking up N over winter, however, is not fully clear until now. Comparable N uptake rates over winter and summer have been reported for some species (Nasholm et al., 2000; Bardgett et al., 2003), but there is also evidence that cold acclimation reduces the potential for N uptake (Malyshev and Henry, 2012a).

Due to increased winter air temperatures, snow cover will decrease in many regions of the temperate zone (Christensen et al., 2007; Kreyling and Henry, 2011). However, air frost events will still occur with unchanged magnitude and duration as nowadays in many temperate regions (Kodra et al., 2011), and with less insulating snow cover, winter soil temperatures can become more variable, particularly in upland and cold temperate regions (Henry, 2008; Brown and DeGaetano, 2011). The resulting more variable soil temperature conditions with frequent soil frost and freeze-thaw cycles (FTC) can affect N cycling. Soil frost and FTC can physically damage plant roots (Tierney et al., 2001) and therefore reduce the plants ability to take up N (Campbell et al., 2014), break up soil aggregates (Oztas and Fayetorbay, 2003), and lyse microbial cells what enlarges the easily available N pool (Skogland et al., 1988), thereby affecting N cycling and leading to N losses in dissolved (Boutin and Robitaille, 1995; Brooks et al., 1998; Joseph and Henry, 2008) or gaseous forms (Matzner and Borken, 2008). For warmer, lowland temperate regions, however, although soil temperature variability might still increase (Kreyling, 2010), an increase in winter air temperatures could lead to fewer soil FTC due to less frost (e.g. lowland Germany, Kreyling and Henry, 2011). Contrasting effects of winter climate change can therefore be expected for colder (stronger effects due to greater increase in soil temperature variability) versus warmer (naturally higher soil temperature variability) temperate regions, and studies of biogeochemical responses to increased soil temperature variability should be designed to account for these differences.

Finally, plant species and vegetation types are known to influence N cycling (Hooper and Vitousek, 1998; Knops et al., 2002). Different plant species and communities further show

different reactions to increased winter temperature variability in the temperate zone, with grasses appearing more responsive than dwarf shrubs (Kreyling et al., 2010; Schuerings et al., 2014) regarding their productivity, probably due to their faster life-cycle. However, this increased responsiveness in productivity of grasses can either be beneficial (Kreyling et al., 2008), or detrimental (Schuerings et al., 2014), probably depending on whether the minimum temperatures experienced after warm phases induce frost damage. Altered plant productivity can therefore indirectly affect N cycling. Generally, stress resistance is linked to nitrogen or nutrient stress tolerance (Macgillivray et al., 1995). Moreover, increased N availability over winter can increase the risk of frost damage to plants (Malyshev and Henry, 2012b).

In this experiment we tested the effects of more variable winter temperature conditions, i.e. recurrent, short winter warming pulses, on soil biotic and potential extracellular enzyme activity, N availability in the soil solution, and N uptake by plants in different plant communities (grassland, heathland; same communities as in Schuerings et al., 2014) at two sites with contrasting winter climate (a warm, snow-poor lowland and a cold, snow-rich upland site). We hypothesised that (1) recurrent winter warming pulses would enhance N-cycling (i.e. increased N availability, soil biotic activity and N uptake into plants). (2) We further expected different responsiveness to the recurrent warming pulses at the two sites, with more variable soil temperatures and stronger frost, therefore frost damage negatively affecting plant N uptake at the colder upland site. (3) Finally, we expected differences among the plant communities in the response of N cycling to the recurrent warming pulses, with a higher ability for winter N uptake in grassland than in heathland plants.

## **2 METHODS**

### **2.1 Experimental design and site description**

This research is part of the EVENT IV experiment, testing the effects of increased winter temperature variability on temperate heath and grassland communities. The effects of the recurrent warming pulses on plant growth (above- and below-ground) are summarized in Schuerings et al. (2014), whereas here we concentrate on nitrogen cycling. The experiment was replicated at two sites: the warm site was located in the Ecological-Botanical Garden of the University of Bayreuth (49° 55' 36.32" N, 11° 34' 57.28" E, 358 m a.s.l.) and the cold site was located at the Waldstein mountain in the Fichtelgebirge (50° 8' 35.81" N, 11° 51' 50.92" E, 781 m a.s.l.). The cold site generally experiences more precipitation and harsher winter conditions (Table 1).

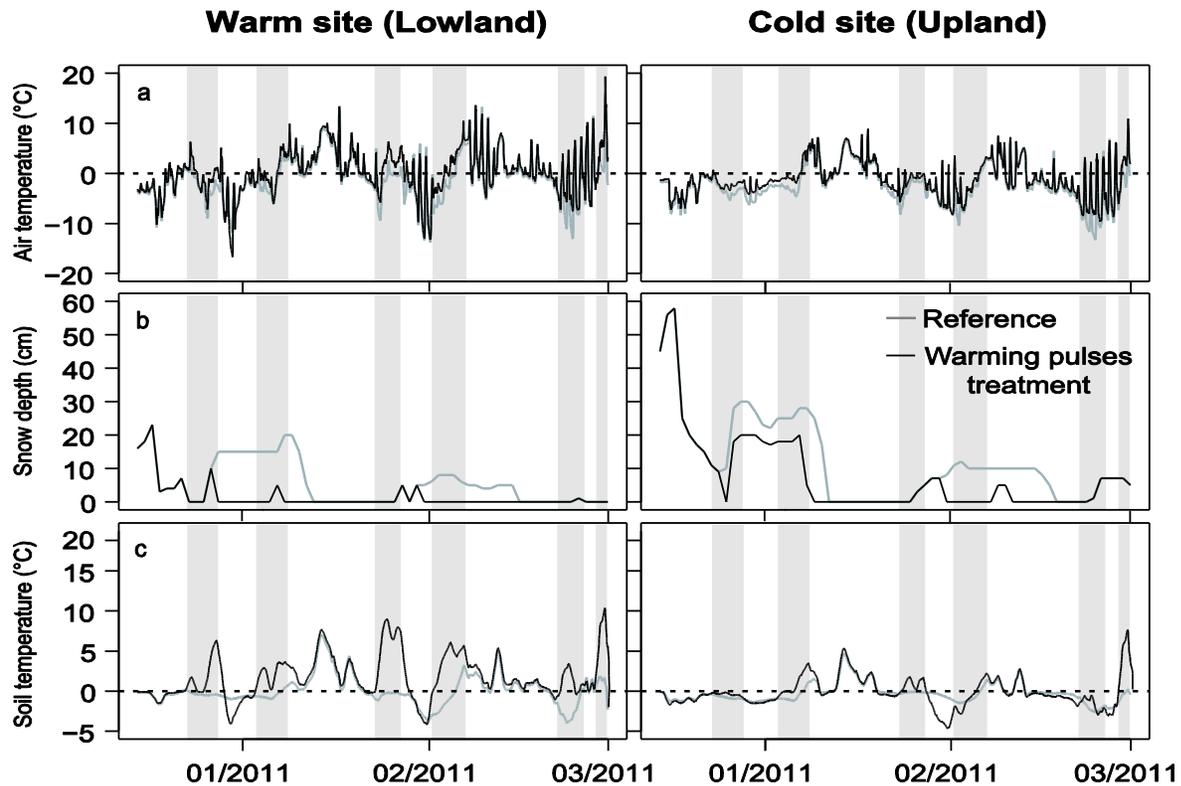
**Table 1:** Climate characteristics of the two experimental sites, measured on site by the department of Micrometeorology until 2008; University of Bayreuth, Prof. T. Foken (Schuerings et al., 2014)

<b>Parameter (Unit; start of measurements warm site / cold site)</b>	<b>Warm site</b>	<b>Cold site</b>
<b>Mean annual temperature (°C; 1998 / 1994)</b>	8.8	5.0
<b>Mean winter temperature (DJF; °C; 1998 / 1994)</b>	0.6	-2.0
<b>Mean annual precipitation (mm; 1998 /1994)</b>	717	1002
<b>Mean winter precipitation (DJF; mm; 1998 / 1994)</b>	158	237
<b>Mean # of days with soil frost (-5 cm) (2003 / 1999)</b>	19	31

The experiment consisted of three fully crossed factors: (1) increased winter temperature variability by application of winter warming pulses versus ambient reference conditions, (2) two experimental sites with naturally different winter climate, (3) six different plant communities and an additional bare ground control. The plant communities consisted of three grassland communities (monocultures of the grass *Holcus lanatus* (L.) and the herb *Plantago lanceolata* (L.), and a community with a mix of both species) and three heathland communities (monocultures of the dwarf shrub *Calluna vulgaris* (L.) and the grass *Deschampsia flexuosa* (L.) and a community with a mix of both species). All species present in this experiment are very common perennial species in Central Europe. In addition, there was a bare ground control in every block. Plant communities were blocked and randomly assigned to the winter warming pulses manipulation and ambient reference. Temperature manipulation blocks, and therefore each factorial combination, were replicated five times. This setup was fully replicated at both experimental sites. For the 140 plots, plastic barrels with 0.2 m<sup>2</sup> surface (50 cm diameter) and 80 cm depth were used as mesocosms. Each of the six mesocosms per treatment was placed in a corner of a hexagon, with 30 cm distance between mesocosms and at least 50 cm separation from the hexagon edge. The bare ground control was placed in the middle of the hexagons. All space between the mesocosms was filled with the same substrate as used within the mesocosms. The soil substrate was homogenized loamy sand (77% sand, 16% silt, 7% clay) from a nearby sand quarry (where all used plant species naturally occur), with a pH=7.35 (measured in 1 M KCl) and a total carbon content of 2.37%. The barrels were attached with outlet hoses at the bottom of each mesocosm, so that the mesocosms functioned as zero tension lysimeters. Sixteen plants per mesocosm were planted in a systematic grid in May 2010. All plants were grown from seed in January 2010, except for the dwarf-shrub *C. vulgaris*, which was obtained as 2-year old individuals in February 2010.

## 2.2 Manipulation of winter temperature variability

Winter warming pulses were applied with six IR-heating lamps (250 W) located in between the mesocosms at a height of 60 cm and surface heating wires (distance 20 cm, 400 W per block), which resulted in 1900 W per block (7 mesocosms). The ambient reference mesocosms were equipped with dummy lamps. Six warming pulses were administered simultaneously for both sites between 15 December 2010 and 28 February 2011 (see Fig. 1).



**Figure 1.** Mean daily air temperature at +5 cm (a), snow depth (b) and mean daily soil temperature at -2 cm (c) at the two experimental sites for the winter warming pulses treatment (black line) and reference conditions (grey line). Warming pulses (grey boxes) were applied between 15th December 2010 and 28th February 2011 (Schuerings et al., 2014).

Warming pulses were administered when there was soil frost at both sites and weather forecast predicted further air frost for at least the next 48 h. Soil temperature (-2 cm; once in every treatment and reference block; 10 measurements per site and 20 in total) and air temperature (+5 cm; one treatment and reference block per site; 2 measurements per site and 4 in total) were measured hourly by thermistors (B57863-S302-F40, EPCOS AG, Germany) connected to a datalogger (dl2, Delta-T Devices Ltd, UK). To quantify the effect of the warming pulses treatment on soil temperature variability, we calculated the coefficient of variation ( $CV =$

standard deviation  $\times$  hourly mean<sup>-1</sup>  $\times$  100; temperatures were converted to K for this). Snow height was measured each morning via a webcam picture of a measuring stick.

### 2.3 Response parameters

Plant available N was measured via the resin stick method (Plant-root-simulator (PRS<sup>TM</sup>)-probes; Western Ag Innovations Inc., Canada). Two cation and two anion PRS<sup>TM</sup>-probes were installed vertically with a distance of 20 cm to each other (0 -15 cm depth) per mesocosm prior to the warming pulse manipulation on 18 December 2010 and collected on 17 March 2011 after the winter warming pulses treatment. PRS<sup>TM</sup>-probes were cleaned and kept in a fridge until being sent to Western Ag Innovations Inc. (Canada) in a cool box for analysis. For the statistical analysis, nitrate and ammonium were pooled due to low ammonium concentrations. The maximum ion capacity of the probes for nitrate is 2088  $\mu\text{g } 10 \text{ cm}^{-2}$ . The values in our study are far lower, showing that the system was not saturated. For better comparability to other studies we give mean plant available N per  $\text{cm}^{-2}$  and day. But it is important to note that N uptake by resin sticks is not a linear process.

Soil biotic activity, i.e. decomposition by microorganisms and feeding by soil fauna, was measured via bait-lamina sticks (terra protecta GmbH, Germany) (Kratz, 1998). One bait-lamina stick containing 16 baits was inserted vertically in the top soil layer of every mesocosm prior to the warming pulses treatment on 18 December. The baits consisted of a mixture of powdered cellulose, bran flakes and active coal. These baits are potentially eaten by earthworms, macro- to micro arthropods and additionally are decomposed by soil microorganisms. The sticks were collected after the winter warming pulses treatment on 17 March, cleaned, and the number of eaten baits was counted. For the latter, sticks were placed on a light bench and when light shined through the baits they were counted as eaten. This analysis was done by a single person who was blind to the factors.

For the potential extracellular enzymatic activity (PEEA), which we used as another proxy for soil biotic activity and decomposition, three soil samples (2 cm diameter, 10 cm depth) per mesocosm were collected and mixed for assays of potential extracellular enzyme activity in soil on 21 February 2011. Soil samples were stored in airtight plastic zip-bags at 4°C and were analysed within 3 days. PEEA assays were carried out with Methylumbelliferone substrates (MUF) (Pritsch et al., 2004; Pritsch et al., 2005). The following PEEAs were measured: MU- $\beta$ -D-glucopyranoside (MU-G), for  $\beta$ -glucosidase, MU- $\beta$ -cellobioside (MU-C) for cellobiohydrolase, MU- $\beta$ -D-xylopyranoside (MU-X) for xylosidase, MU-phosphate (MU-P) for acid phosphatase. Substrates and calibration saturation and incubation times were determined in pre-experiments (data not shown) as follows: MU-G and MU-X each 500  $\mu\text{M}$  incubating for 60 min, MU-C 500  $\mu\text{M}$  incubating for 120 min, MU-P 800  $\mu\text{M}$  incubating for 40 min. Fluorescence was detected at an excitation wavelength of 360 nm and an emission wavelength of 450 nm with a Gemini EM Fluorescence Microplate Reader from Molecular Device, California.

Prior to the warming pulses treatment (18 December 2010), plots were labelled with 0.02 g Potassium Nitrate-<sup>15</sup>N (min. 99.19 atom % <sup>15</sup>N; Campro Scientific GmbH, Germany), dissolved in 250 ml deionized water, resulting in 0.1 g <sup>15</sup>N m<sup>-2</sup>. Leaf (2-3 medium aged leaves per plot and species, randomly chosen), root (fine roots from a soil sample taken directly next to a randomly chosen plant per mesocosm and species) and soil samples (3 soil samples per plot were mixed; 2 cm diameter, 10 cm depth) were taken on 17 March 2011, after the winter warming pulses treatment. The samples were kept frozen until they were cleaned, dried (48 h at 50° C) and ball milled. Mass spectroscopy analysis was done at the laboratory of Isotope Biogeochemistry, BayCEER, University of Bayreuth, with a combination of an elemental analyzer (Carlo Erba NC 2500, CE Instruments, Italy) and an isotope mass spectrometer (delta plus, Thermo Fisher Scientific, Germany). Atom % increase values for plant and soil material collected after the winter warming pulses treatment were calculated by comparing to values obtained from unlabelled reference plants (n = 5 per species) and soil material taken prior to the winter warming pulses treatment (n = 3 per experimental site). Due to missing volume readings, the isotopic signature of leachate could only be determined and related to volume of leachate for four mesocosms (*Holcus lanatus* and *Plantago lanceolata* mixed mesocosms at both sites for both winter warming pulses treatments), which were permanently equipped by tipping buckets (7041.3000X, Theodor Friedrichs & Co., Germany). Therefore, no mass balancing of the label was possible, and we report <sup>15</sup>N-atom% here. For interpretation of the data it is important to note that overall above-ground biomass significantly decreased by 9.2 % due to the warming pulses treatment (Schuerings et al., 2014). For single species, only *H. lanatus* showed a strong decrease by 29.2 % whereas the other species showed no significant treatment effects (Schuerings et al., 2014).

## 2.4 Data analyses

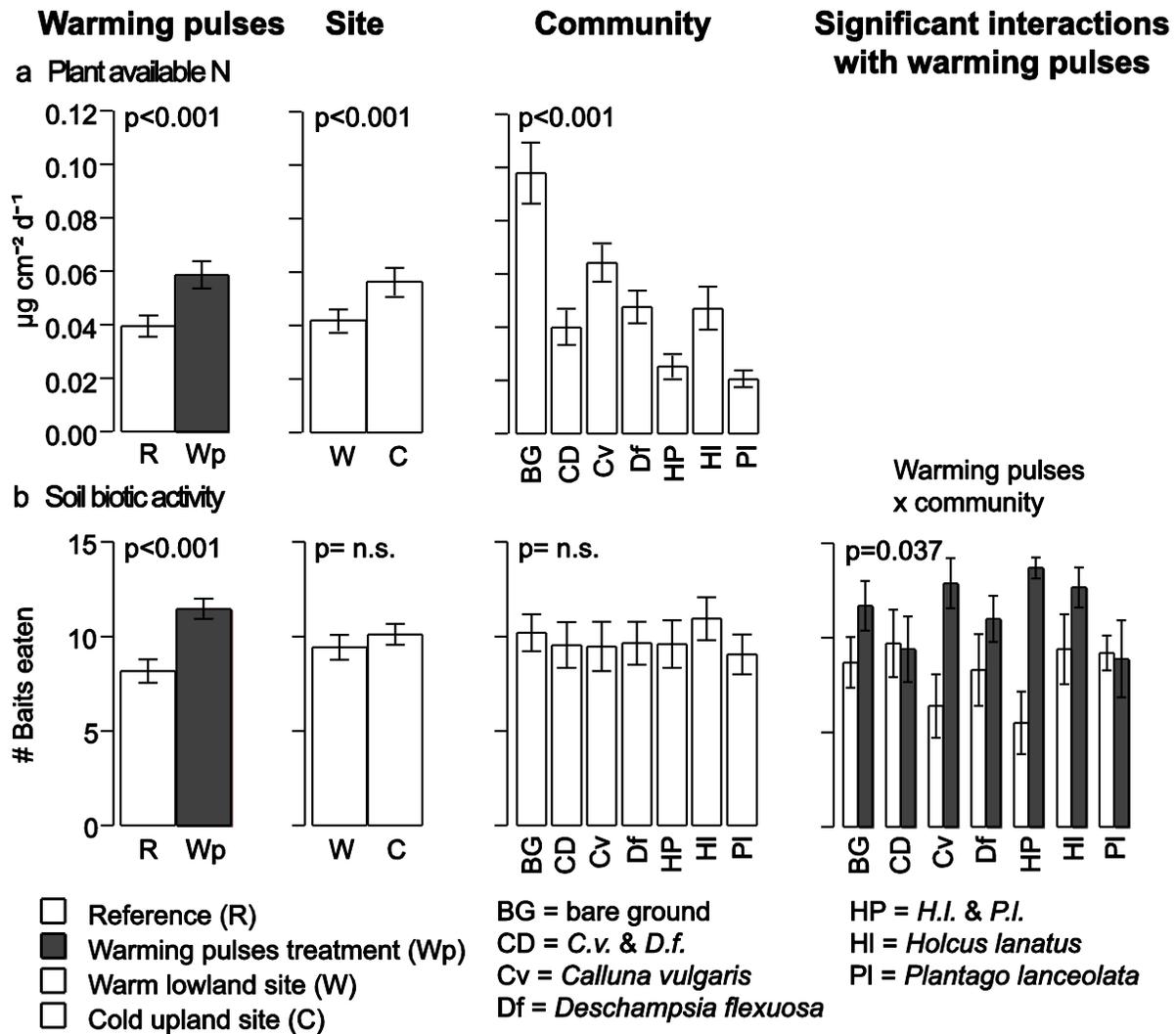
Linear mixed-effect models combined with analysis of variance (ANOVA) were applied to test for significant winter warming pulses treatment, site and plant community effects. All possible interactions of community or species and site with the warming pulses treatment were included as fixed effects (s. Table 2 & 3 for all tested interactions). For the analysis of <sup>15</sup>N content in plants, species identity was included as a fixed factor instead of community composition, whereas community was included as a random effect. Block identity was set as a random effect in all models, thereby accounting for the blocked design. Before statistical analysis, we tested for normality and homogeneity of variance by examining the residuals versus fitted plots and the normal qq-plots of the linear models (Faraway, 2005). If conditions were not satisfactorily met, we applied log(x)- (plant available N; <sup>15</sup>N atom% increase of leaves and roots; PEEA of beta-glucosidase, cellobiohydrolase, xylosidase), log(x+1)- (<sup>15</sup>N atom% increase in soil), or sqrt(x)- (PEEA acid phosphatase) transformation. Significance level was set to p < 0.05. All statistical analyses were performed using R 2.12.2 (R Development Core Team 2011) and

additional packages nlme (Version 3.1-98, 2011) and sciplot (Version 1.0-9, 2011) for graphical illustrations.

### 3 RESULTS

The winter warming pulses manipulation successfully decreased snow cover and resulted in increased soil temperature variability (Fig. 1). At the warm site, variation in soil temperature during the manipulation period (15 December 2010 to 28 February 2011) was increased to CV = 0.99 in comparison to CV = 0.66 in the reference mesocosms. Mean soil temperature increased to 1.8°C in the manipulation as compared to 0.1°C in the ambient reference. Minimum temperature reached -4.2 °C and -4.0 °C, respectively. For the cold site, variation in soil temperature during the manipulation period increased to CV= 0.68 in comparison to CV= 0.43 in the reference mesocosms. Mean soil temperature was almost unchanged with -0.1°C in the warming pulses manipulation and -0.3°C under ambient reference conditions. However, minimum temperature was considerably lower in the warming pulses mesocosms, reaching -4.7 °C, as compared to -2.6 °C in the reference mesocosms. The number of soil freeze thaw cycles was not altered noticeably at any site (warm site: 7 vs. 8, cold site: 6 vs. 5).

Plant available nitrate and ammonium significantly increased by 34.5% in response to the winter warming pulses treatment (F=13.5, p<0.001; Table 2, Fig. 2). The cold site overall had a 48.4% higher amount of N available than the warm site (F=20.0, p<0.001; Table 2, Fig. 2). Plant community composition also influenced plant available N (F=18.4, p<0.001; Table 2, Fig. 2). Bare ground control mesocosms had the highest N values, followed by the heathland communities and then the grassland communities, with only monocultures of *H. lanatus* reaching levels of the heathland communities. Winter warming pulse effects were not influenced by site or plant community (no significant interactions, Table 2).



**Figure 2.** (a) Plant available nitrogen (nitrate and ammonium; PRS<sup>TM</sup>-probes) and (b) soil biotic activity (bait-lamina test) during the manipulation period (18 December 2010 - 17 March 2011). Main winter warming pulses treatment, site and community effects and all significant interactions between the winter warming pulses treatment with site and community are shown. Mean ( $\pm$  S.E.) values are shown ( $n=140$ ).

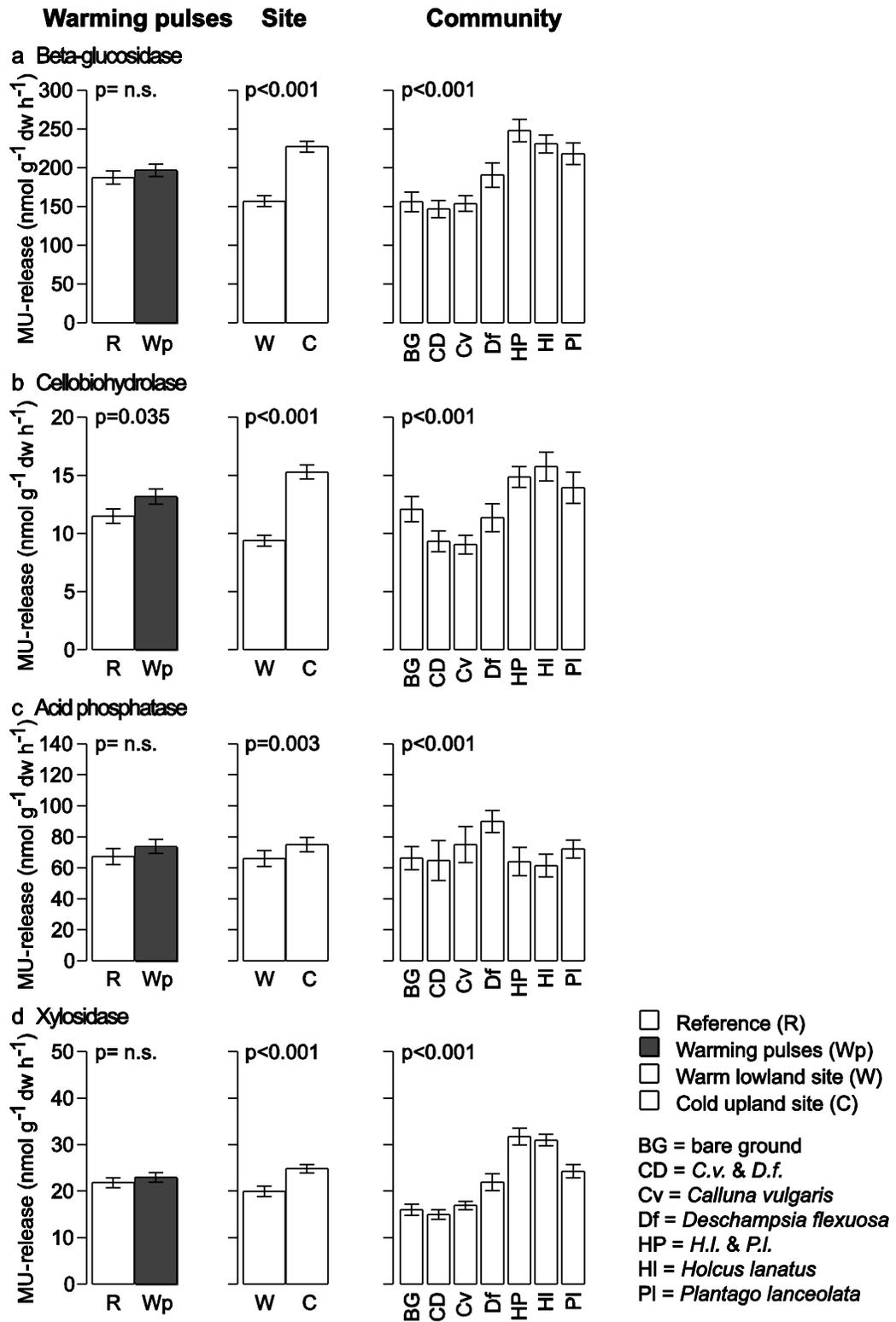
**Table 2:** ANOVA-results of all tested main and interaction effects for N mobilization, i.e. N availability in the soil solution ( $\text{NH}_4^+$  and  $\text{NO}_3^-$ ), soil biotic activity (bait-lamina test), and the four tested potential soil enzyme activities. Warming pulses: Winter warming pulses treatment.

Factor	N availability in soil solution		Soil biotic activity		Beta-glucosidase activity		Cellobiohydrolase activity		Acid phosphatase activity		Xylosidase activity	
	F	P	F	P	F	P	F	P	F	P	F	P
Warming pulses	<b>13.5</b>	<b>&lt;0.001</b>	<b>17.5</b>	<b>&lt;0.001</b>	1.8	0.199	<b>5.3</b>	<b>0.035</b>	2.6	0.127	2.0	0.173
Site	<b>20.0</b>	<b>&lt;0.001</b>	0.6	0.441	<b>67.2</b>	<b>&lt;0.001</b>	<b>69.2</b>	<b>&lt;0.001</b>	<b>12.6</b>	<b>0.003</b>	<b>33.6</b>	<b>&lt;0.001</b>
Community	<b>18.4</b>	<b>&lt;0.001</b>	0.3	0.912	<b>23.5</b>	<b>&lt;0.001</b>	<b>16.2</b>	<b>&lt;0.001</b>	<b>32.5</b>	<b>&lt;0.001</b>	<b>44.5</b>	<b>&lt;0.001</b>
Warming pulses x Site	0.6	0.425	0.9	0.358	3.2	0.094	1.3	0.266	0.9	0.359	3.8	0.068
Warming pulses x Community	0.2	0.961	<b>2.3</b>	<b>0.037</b>	1.4	0.213	0.7	0.663	1.1	0.388	0.6	0.694
Warming pulses x Site x Community	0.6	0.715	1.1	0.370	0.7	0.685	1.0	0.400	0.9	0.500	1.4	0.212

Soil biotic activity, i.e. the number of eaten baits, increased by 40% ( $F=17.5$ ,  $p<0.001$ ; Table 2, Fig. 2) due to the winter warming pulses treatment in comparison to reference conditions. Soil biotic activity did not significantly differ between sites or plant communities. The warming pulses effect, however, was influenced by the plant communities ( $F=2.3$ ,  $p=0.037$ ), with slightly decreasing activities in monocultures of *P. lanceolata* and mixed communities of *C. vulgaris* & *D. flexuosa* due to the warming pulses (Fig. 2). All other communities showed an increase in soil biotic activity due to the warming pulses. No other interaction with the warming pulses treatment yielded significance for soil biotic activity (Table 2).

Regarding PEEA there was a general trend towards higher values under the winter warming pulses treatment, yet only for cellobiohydrolase was this effect statistically significant ( $F=5.3$ ,  $p=0.035$ ). For the other three tested enzymes no significant effect of the winter warming pulses treatment was observed. Generally, there were significantly higher PEEAs at the cold site than at the warm site (Table 2, Fig. 3) and plant community composition effects differed such that, except for acid phosphatase, grassland communities showed higher PEEA than heathland communities (Table 2, Fig. 3). No significant interactions between the warming pulses treatment and site or plant community were observed (Table 2).

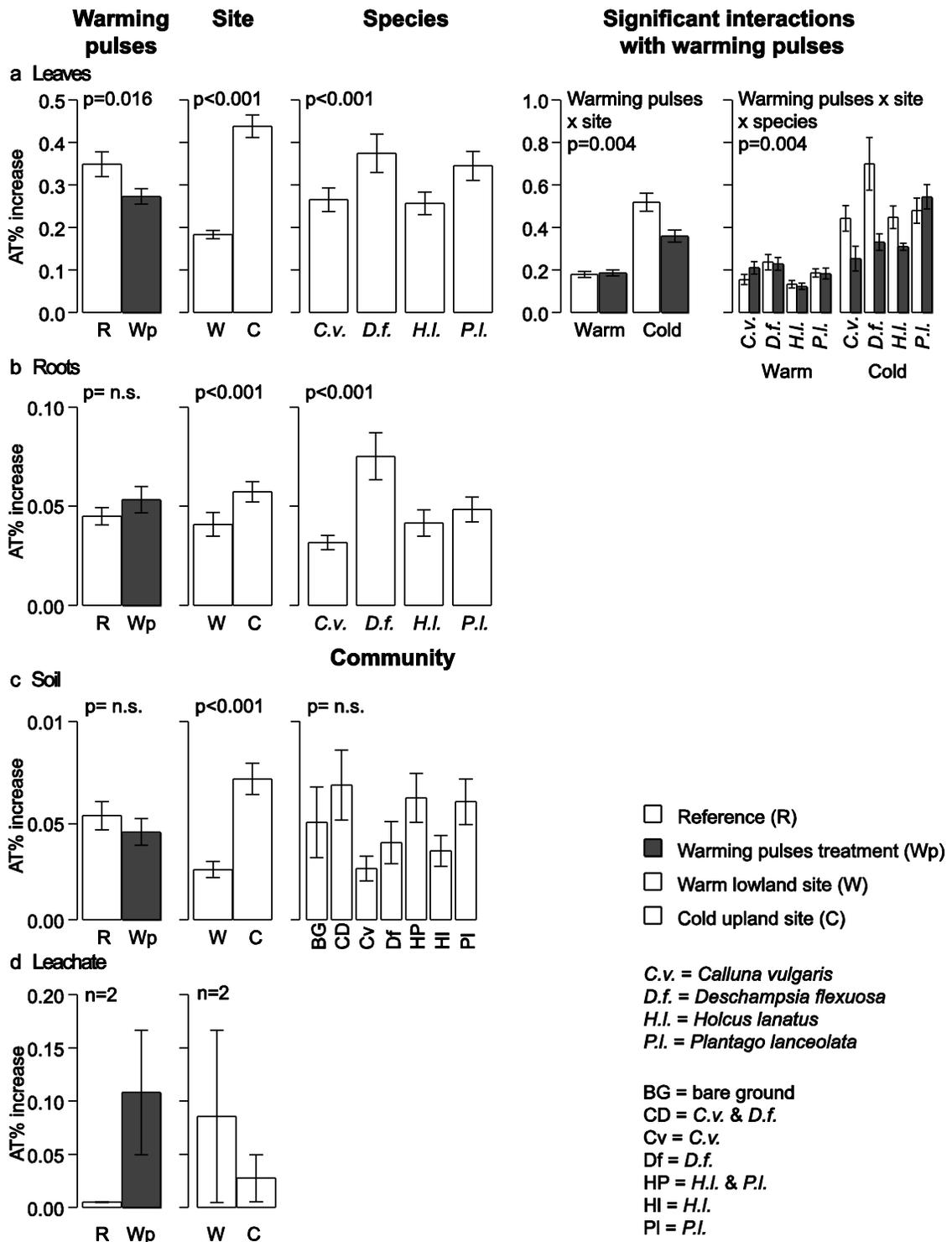
The AT%  $^{15}\text{N}$  values in leaves were significantly reduced by 21.7% (relative difference) under the winter warming pulses treatment in comparison to reference conditions ( $F=5.9$ ,  $p=0.016$ ), whereas for root and soil material no significant winter warming pulse effect was observed (Table 3, Fig. 4). For leachate, no statistical analysis was performed due to the low replication, but for the existing samples ( $n=2$  per winter warming pulses treatment), a clear trend towards increased leaching of the  $^{15}\text{N}$ -tracer was observed (Fig. 4). Generally, the cold site showed significantly higher plant AT%  $^{15}\text{N}$  values than the warm site (Table 3, Fig. 4). *D. flexuosa* exhibited the highest AT%  $^{15}\text{N}$  values, followed by *P. lanceolata*, with the same pattern observed for leaves and roots. Significant decreases in the  $^{15}\text{N}$  signal in plant leaves (-30.7%) in response to warming pulses only occurred at the cold site (winter warming pulses treatment x site interaction:  $F=8.6$ ,  $p=0.004$ ; Table 3, Fig. 4). The significant three-way interaction between warming pulses treatment, site, and species identity ( $F=3.4$ ,  $p=0.004$ ) indicated that the decrease in  $^{15}\text{N}$  values only happened at the cold site and only for three of the four species (*C. vulgaris*, *D. flexuosa* and *H. lanatus*; Fig. 4).



**Figure 3.** Mean potential soil enzymatic activity for the four tested enzymes (a)  $\beta$ -glucosidase, (b) cellobiohydrolase, (c) acid phosphatase and (d) xylosidase (all  $\pm$  S.E.) during the manipulation period (18 December 2010 - 17 March 2011). Main winter warming pulses treatment, site and community effects are shown. No significant interactions between the winter warming pulses treatment with site and community were detected.

**Table 3:** ANOVA-results of all tested main and interaction effects for the fate of a  $^{15}\text{N}$  label (increase in atom %  $^{15}\text{N}$  in the compartments leaves, fine roots, and bulk soil). Warming pulses: Winter warming pulses treatment.

Factor	$^{15}\text{N}$ atom % increase					
	Leaves		Roots		Bulk soil	
	F	P	F	P	F	P
Warming pulses	<b>5.9</b>	<b>0.016</b>	1.5	0.228	0.9	0.331
Site	<b>144.5</b>	<b>&lt;0.001</b>	<b>19.3</b>	<b>&lt;0.001</b>	<b>29.9</b>	<b>&lt;0.001</b>
Species/Community (Soil)	<b>7.4</b>	<b>&lt;0.001</b>	<b>9.6</b>	<b>&lt;0.001</b>	1.7	0.134
Warming pulses x Site	<b>8.6</b>	<b>0.004</b>	2.1	0.153	2.0	0.162
Warming pulses x Species	1.2	0.313	0.5	0.695	0.7	0.647
Warming pulses x Site x Species	<b>3.4</b>	<b>0.004</b>	1.0	0.422	1.2	0.292



**Figure 4.** Mean increase in atom% values ( $\pm$  S.E.) for leaves (n=80), roots (n=80), bulk soil (n=70) and leachate (n=2). Before the warming pulses treatment all plots were watered with 0.25 l of water with 0.02 g Potassium Nitrate- $^{15}\text{N}$  (min. 99.19 atom %  $^{15}\text{N}$ ). Main winter warming pulses treatment, site and community effects and all significant interactions between the winter warming pulses treatment with site and community are shown. It is important to note that total above-ground biomass declined by 9.2 % in the growing season after manipulations, so that tracer dilution effects due to increasing biomass can be excluded (Schuerings et al., 2014).

## 4 DISCUSSION

Recurrent winter warming pulses led to increased soil temperature variability and influenced N cycling in our experiment. As expected, N availability was increased (+35%) in the mesocosms which received the winter warming pulses treatment. Increased N availability during winter/early spring is often explained by freeze-thaw events resulting in increased biological and physical decomposition of soil organic matter (SOM) (Matzner and Borken, 2008) and increased N mineralization (Rustad et al., 2001; Melillo et al., 2002). Yet, in our study FTC frequency was merely changed between winter warming pulses and references plots ( $\pm 1$ ), implying that the warming pulses treatment affected N availability either through increased temperature variability or the increase in mean temperature. Due to the winter warming pulses soil biotic activity increased by 40%. This increase in soil biotic activity is in line with results from other winter warming experiments which measured soil respiration as an index of soil biotic activity (Davidson and Janssens, 2006; Allison and Treseder, 2011). The soil enzymes we examined play a major role in the decomposition of biological material (Marx et al., 2001).

We observed significantly increased PEEA for cellobiohydrolase, whereas for the other three tested enzymes the observed increases were not significant. Therefore in our experiment, increased soil temperature variability led to increased biotic decomposition as indicated by increased soil biotic activity and increased PEEA of cellobiohydrolase. In winter warming experiments, increased N cycling is often attributed to changes in the frequency of soil FTC (Mikan et al., 2002). Despite only small changes in FTC frequency in our mesocosms, however, we observed increased N availability, increased soil biotic and soil potential enzymatic activity. However, for the cold site, where it is important to note that mean soil temperature only increased by 0.2 °C, mean minimum temperature was considerably lower in the warming pulses mesocosms, reaching -4.7 °C, as compared to -2.6 °C. Since we found lowered N incorporation into plants (see discussion further down) and stable or lower plant biomass (Schuerings et al., 2014) at the cold site, this could have lowered N immobilization by plants. The temporal dynamics of soil temperature, in particular the intensity of freezing right after warming pulses, is therefore another important determinant of N cycling responses, possibly leading to frost damaging of dehardend plants. While changed FTCs (Joseph and Henry, 2008), warmer mean soil temperatures (Rustad et al., 2001; Melillo et al., 2002) and single extreme frost events (Elliott and Henry, 2009) are known to be important drivers of N cycling, our results imply that soil temperature variability, i.e. temperature dynamics, can also affect N availability and soil biotic activity.

We found significantly higher N availability and potential activity of all four tested potential soil enzymes for the cold site despite lower mean temperatures at the site. Groffman et al. (2009) found the same pattern along an altitudinal gradient in a northern hardwood forest. This suggests that the local climate may have an important influence on the magnitude of N mobilization processes. However, since we found no significant interaction between winter

warming pulses treatment and site, the effects of winter warming pulses on N availability, soil biotic activity and potential soil enzymatic activity therefore appear independent of the local climate.

The mobilization of N was influenced by the plant community composition, with the bare ground control showing highest levels of available N. Since there were no roots in the bare ground plots competing with the PRS<sup>TM</sup>-probes for N, this result is not surprising. Regarding plant communities, there was no clear pattern in N availability, although the heathland communities showed higher values than grassland communities with the exception of monocultures of *H. lanatus*, which showed similar values as the heathland communities. The interaction between the warming pulses treatment and plant community indicated that plant species composition influenced soil biotic activity differently under winter warming pulses. However, there was no clear pattern, since all communities showed increased soil biotic activity in response to the winter warming pulses, except for monocultures of *P. lanceolata* and mixed cultures of *C. vulgaris* and *D. flexuosa*. Potential soil enzymatic activity was generally higher in grassland mesocosms in comparison to heathland mesocosms, with the exception of acid phosphatase.

The <sup>15</sup>N signal in plants leaves was, contrary to our expectations, decreased by the winter warming pulses treatment. Plants can lose their cold hardiness within hours in response to elevated temperatures (Kalberer et al., 2006), and subsequent frost events after a winter warm spell can thus damage plants substantially (Bokhorst et al., 2009). Freezing intensity is also an important determinant of plant frost damage, and while most temperate species can tolerate temperatures at or below freezing, there is often a threshold subfreezing temperature where damage intensifies (Malyshev and Henry, 2012a). Notably, the minimum temperatures reached in the reference mesocosms at the cold site were the least severe, and the highest AT% <sup>15</sup>N values were observed in these plots, whereas minimum soil temperatures of at least -4 °C were reached in the treatment plots at the cold site and in all of the warm site mesocosms, all of which featured relatively low <sup>15</sup>N values. Similarly, in other systems, grass ecotypes located at northern sites that are protected from cold air by thick snow cover have developed lower frost tolerance than conspecific ecotypes located in warmer locations that feature less snow cover, because the latter ecotypes experience more intense frost (Dionne et al., 2010).

We also observed significant differences among the tested species in the increase of AT% <sup>15</sup>N values, which is not surprising, given that species exhibit wide variation in their nutrient uptake capacities (Hooper and Vitousek, 1998; Knops et al., 2002). The interesting point is that the reduction in <sup>15</sup>N values only happened at the cold site and only for *C. vulgaris*, *D. flexuosa* and *H. lanatus* (interaction: winter warming pulses treatment x site x species). Total above-ground biomass of all tested species decreased by 9.2 % in response to the winter warming pulses treatment (Schuerings et al., 2014), thus dilution effects on N-tracer uptake can be excluded. Lower or stable above-ground biomass and lower AT% <sup>15</sup>N values combined are a clear hint for

reduced N uptake by the affected plant species. Such differences among species in frost susceptibility could have important consequences for competitive balances and shifts in community composition over the long term (Joseph and Henry, 2008; Cornelissen and Makoto, 2014).

Chronic winter warming can increase above-ground biomass (Hutchison and Henry, 2010; Natali et al., 2012; Schuerings et al., 2013). This additional growth may be fuelled by increased N mobilization in early spring. Pulsed winter warming increasing the risk of frost damage, however, complicates this simple expectation of increased plant growth under winter climate change. The inability of frost-damaged plants to take up the available N in the soil solution might trigger N losses from ecosystems by N leaching or gaseous losses (Ineson et al., 1998; Campbell et al., 2014). In this experiment we also found species-specific responses in above-ground biomass production due to the winter warming pulses (Schuerings et al., 2014); only *H. lanatus* showed a decrease in above-ground biomass, whereas the other tested species remained unaffected by the winter warming pulses treatment in their above-ground productivity. Taken together, species- or vegetation type-specific responses have to be taken into account when forecasting effects of climate change on N-cycling (Makoto et al., 2014). Furthermore, regarding winter climate change, pulsed warming events can result in opposing effects on N cycling and biomass accumulation than chronic warming.

## 5 CONCLUSIONS

Future winters in the temperate zone are expected to be characterized by more variable soil temperatures due to increasing air temperature variability and due to missing insulation by snow. Our experiment implies that more variable soil temperatures enhance nitrogen mobilization in the soil independent from vegetation types and the local climate. Plant performance, however, depended on local climate, with plant  $^{15}\text{N}$  immobilization during winter and early spring after exposure to winter warming pulses being reduced at colder sites, probably due to frost damage after the warming pulses. This pattern implies increased risk for nitrogen leaching at colder temperate sites in response to increased winter temperature variability. Taken together, our findings emphasize the importance of temperature variability, plant performance, and frost damage in a warmer world for nitrogen cycling and nitrogen losses from ecosystems.

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## Manuscript 7: Absence of soil frost affects plant-soil interactions in temperate grasslands

**Plant and Soil 2013. 371: 559-572**

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Keywords: winter warming, climate change, freeze-thaw, plant productivity, nutrient cycling, soil biotic activity

### ABSTRACT

*Background and aims:* Intermittently frozen ground in winter is expected to disappear over large areas in the temperate zone due to ongoing climate warming. The lack of soil frost influences plant soil interactions and needs to be studied in more detail.

*Methods:* Winter soil frost was avoided by belowground heating wires in a field experiment over two subsequent winters in a temperate grassland. Soil respiration, soil nitrogen availability and plant performance (aboveground biomass, root length at two depth levels, greenness, nutrient content) were compared between “no-frost” and reference plots which underwent repeated freeze-thaw cycles in both winters.

*Results:* Soil respiration increased in the “no-frost” treatment during the warming phase (+291 %). N-availability in the upper 10 cm of the soil profile was not affected, possibly due to

increased plant N accumulation during winter (+163%), increased plant N concentration (+18%) and increased biomass production (+31.5%) in the growing season. Translocation of roots into deeper soil layers without changes in total root length in response to the “no-frost” treatment, however, may be a sign of nutrient leaching.

*Conclusions:* The cumulative effect on carbon cycling due to warmer soils therefore depends on the balance between increased winter carbon loss due to higher soil biotic activity and enhanced plant productivity with higher nutrient accumulation in the growing season.

## INTRODUCTION

Climate is changing, with observed warming over the last 30 years being greatest at higher northern latitudes and in winter (Christensen et al. 2007). In Germany, winter temperatures increased by 2.3° C between 1981 and 2000 (German Weather Service). Due to ongoing global warming, regions with no or rare soil frost are going to expand (Kreyling and Henry 2011). A lack of soil frost could directly or indirectly lead to changes in carbon (C) and nutrient cycling, with implications for ecosystem functioning such as decomposition, primary production or carbon sequestration. However, knowledge about the effects of soil frost absence on plant-soil-interactions is sparse, particularly in the temperate zone (Kreyling 2010).

As temperature is an important driver for metabolic reactions, absent soil frost in winter can increase soil biotic activity if soil moisture does not become limiting (Davidson and Janssens 2006; Allison and Treseder 2011). While soil frost reduces the CO<sub>2</sub> emissions via soil respiration, thawing of frozen soil can lead to strong CO<sub>2</sub> pulses from the soil (Muhr et al. 2009). Carbon losses from soils that are currently exposed to extended frost periods are expected to increase as the soil frost duration subsides and increased soil biotic activity accelerates soil respiration and C loss from ecosystems (Rustad et al. 2001; Melillo et al. 2002). Generalisation across systems and temporal extent of such reactions, however, are unclear (Luo et al. 2001; Melillo et al. 2002; Wan et al. 2007).

Soil C and nitrogen (N) concentrations increase after soil freeze-thaw cycles (FTC) due to microbial lyses, death of roots, and changes in soil structure (Matzner and Borken 2008). Soil

warming also increases N mineralization (Rustad et al. 2001; Melillo et al. 2002). Many plants remain photosynthetically active in winter (Larsen et al. 2007) and are capable of winter N uptake (Laine et al. 1994; Grogan et al. 2004; Andresen and Michelsen 2005; Malyshev and Henry 2012). It is unclear whether N mobilization due to increased mineralization will lead to N-leaching and loss from the ecosystem, or if the vegetation is capable of increased winter N uptake resulting in increased primary production (Ineson et al. 1998; Kreyling et al. 2008).

Observations of ecosystems in northern latitudes show an earlier start and increase in photosynthetic activity in spring with rising temperatures (Zhou et al. 2001; Loik et al. 2004). Greenness was used as a surrogate for photosynthetic activity in this study. Continuous winter air warming increases aboveground net primary production (ANPP) (Hutchison and Henry 2010; Kardol et al. 2010). Likewise winter soil warming pulses leading to additional FTC have been shown to increase ANPP in temperate grasslands (Kreyling et al. 2008). However, as plants become more active over winter they also lose frost hardiness, making them vulnerable to frost events in winter (Bokhorst et al. 2009) or spring (Kreyling et al. 2012). Root length decreases with winter soil warming pulses (Kreyling et al. 2008), likely due to frost damage to dehardened plant tissue while warming throughout winter increases root length (Hutchison and Henry 2010). Artificial spring soil warming prior to a natural spring thaw in a boreal forest also leads to an increase in root length (Majdi and Ohrvik 2004). The role of soil frost and freeze-thaw events versus the role of warmer mean soil temperatures remains unclear, however. No studies to our knowledge have administered warming to a level where soil frost does not occur at all.

Warmer soils increase soil enzyme activities leading to higher soil organic matter decomposition and changing N, P and K availabilities (Sardans et al. 2012a, 2012b), potentially leading to higher plant nutrient accumulation and changing stoichiometric relationships. Changes in microbial community, root length and in soil structure affect plant-soil nutrient cycles by variable solubility and chemical traits of the respective elements. Warming and drought have been proven to asymmetrically affect soil nutrient status (e.g. Sardans et al. 2008a) and plant elemental composition (e.g. Sardans et al. 2008b, 2008c) in Mediterranean ecosystems as well as in other biomes (Sardans and Peñuelas 2012). Changes in stoichiometric relationships can change plant metabolome, production and growth rate in turn affecting ecosystem structure and function (Rivas-Ubach et al. 2012; Sardans et al. 2012a). The kinds of plant species present also modifies

plant elemental composition because each plant species tends to have a particular elemental composition such as projected by the biogeochemical niche hypothesis (Peñuelas et al. 2008). Different shifts in species biogeochemical niche have been observed under climate change (Peñuelas et al. 2008). However, very little is known about the effects of winter warming on nutrient cycling and stoichiometry in plant-soil systems (Sardans and Peñuelas 2012).

Here, we investigated how the absence of winter soil frost affected plant-soil interactions in two artificial temperate grassland communities over two winters and into the following growing seasons. We hypothesised that the absence of soil frost would lead to (1) increased soil biotic activity in winter, leading to (2) increased nutrient availability, and consequently to (3) increased winter activity of plants. The increase in plant winter activity should lead to earlier greening, higher ANPP and increased root growth. We further hypothesised that changes in microbial community, root length and soil structure due to absence of soil frost would (4) asymmetrically affect different nutrients and different species, thereby producing changes in plant elemental concentrations and stoichiometry.

## **MATERIALS AND METHODS**

### **Experimental design and site description**

The research is part of the EVENT I -experiment (Jentsch et al. 2007) where the effects of climate change such as drought, heavy rain or winter warming on temperate plant communities are studied. The experimental site is located at the Ecological-Botanical Garden of the University of Bayreuth, Germany (49°55'19"N, 365 m asl). Mean annual air temperature at the site is 8.2°C and mean annual precipitation is 724 mm (data: German Weather Service, 1971 – 2000). With average January air temperatures of -1.0°C, the site is located at the transition between oceanic and continental climates. Winter soil frost depends on site conditions in the vicinity of the experimental site: early snow-pack or energy fluxes from ascending ground water can prevent soil frost completely, while well-drained, open sites such as our experimental site may freeze for several weeks.

All plots consisted of homogenized soil of 80 cm depth including 20 cm topsoil. The soil was taken from a nearby sand quarry. Topsoil carbon content totalled 2% and pH = 4.5

(measured in 1 M KCl), whereas the lower soil layer had 0.2% total carbon and pH = 6.2. The texture of the soil body was loamy sand (82% sand, 13% silt, 5 % clay). Bulk density for both soil layers was 1.6 g cm<sup>-3</sup>.

In the “no frost”-treatment, soil temperature was manipulated by buried heating wires (deviflex DTIP, DEVI, Vejle, Denmark) to avoid soil frost completely. The wires were located at a depth of 7 cm and 20 cm apart from each other, resulting in 100 W m<sup>-2</sup>. Installation was finished in the year prior to planting. Soil temperature manipulations were conducted from 1 December to 28 February during the winters 2009/2010 and 2010/2011. The reference plots did not receive any treatments. An artefact control with heating wires installed the same way as in our “no-frost” plots showed no difference for plant growth in comparison to untreated controls at the same site in a previous experiment (Kreyling et al. 2008).

Grassland communities of two different functional compositions were studied: one community consisted of two grasses and thus only one plant functional group (*Arrhenatherum elatius* and *Holcus lanatus*) (grasses-only), whereas the other community consisted of the same two grass species and two additional herbs (*Geranium pratense* and *Plantago lanceolata*) (grasses&herbs).

The plant communities were blocked and randomly assigned within the “no-frost” and reference plots. Each factorial combination was replicated five times resulting in 20 plots (2 x 2 m in size). The plants were grown from seeds in autumn 2004 and planted in April 2005. One hundred individuals per plot were planted in a hexagonal grid with a distance of 20 cm between neighbours. All species are perennial and original composition was maintained by periodical weeding. An analysis of species compositions (Kreyling et al. 2011) and above- and belowground biomass (Kreyling et al. 2010) showed no significant difference between the treatments prior to the first soil warming manipulation in winter 2009/2010.

## Response parameters

Soil (-2 cm) and air temperature (+5 cm) were measured hourly in every plot by thermistors (B57863-S302-F40, EPCOS) connected to a datalogger (dl2, Delta). Snow height was manually measured each morning.

Soil respiration was measured biweekly or monthly (30 dates) from 22 March 2010 until 11 April 2011 on each plot of the four species community. Measurements were carried out with a respiration chamber connected to a non-dispersive infrared gas analyzer (SPC-1 & EGM-4, PP-systems, USA). The respiration chamber was placed on PVC-collars to get a closed system. The collars (10 cm in diameter, 5 cm in height) were installed into the soil one month before the start of measurements at a depth of 4 cm. The day before each measurement all aboveground plant material was clipped from the collar. The CO<sub>2</sub> fluxes were measured for four minutes, only analysing the last soil respiration rate values. Mixed soil samples of the upper layer (0-10 cm) of every plot were taken on six dates to quantify plant-available nitrate and ammonium content over winter and into spring (17 December 2009, 20 January 2010, 18 February 2010, 24 March 2010, 11 March 2011 and 23 March 2011). The samples were sieved (2 mm), extracted to a 1 M KCl solution and then filtered (Roth, Typ 15 A Blauband). Quantification was done by flow injection analysis (FIA, measurements conducted at BayCEER Analytical Chemistry, Bayreuth, device: MLE Dresden FIA-LAB).

To measure plant activity early in the growing season, phenology of greenness was quantified by digital pictures, taken under standardized light conditions biweekly from 1 March 2011 till 14 April 2011. For this purpose, a portable light-tight box (56 x 55 x 75 cm) with a camera (Nikon D2x) and artificial lighting (a flash) was used. The calculation of the greenness was based on Marchand et al. (2004), using a transformation from the RGB-photos to the HSL colour space. The determination of threshold values of the HSL-bands for the “greenness” was performed with remote sensing software ENVI 4.7 (Exelis Visual Information Solutions, Boulder, Colorado, USA) and ArcGIS 10 (Environmental Systems Research Institute, Redlands, California, USA). The processing and calculation of the percentage of greenness was done with the same parameters for all photos and all time steps with ImageMagick 6.7.6-5 (ImageMagick Studio LLC, Landenberg, Pennsylvania, USA).

Above-ground net primary productivity (ANPP) was measured by complete above-ground harvests of the central 1m<sup>2</sup> of the plots. Harvests were done on 28 June 2010, 13 September 2010 and on May 26 2011. Harvested biomass was sorted by species, dried to constant weight at 70°C and weighed.

A minirhizotron technique was used to determine root length. A clear plastic tube (5 cm diameter) was installed at a 45° angle to a depth of 45 cm in each plot before planting. Above-ground parts of the tubes were covered with adhesive aluminium foil and the tubes were capped to prevent entry of water, dust, light and heat. Images of 4 cm<sup>2</sup> were taken at a depth of 5 and 15cm with a digital camera (Nikon Coolpix E995) mounted on an endoscope. A line intersection method (Tennant 1975) within a systematic grid of 10 x 10 (grid unit: 0.2 cm x 0.2 cm) was used to quantify root length. Sampling was done on 20 April 2010, 30 June 2010, 17 September 2010 and 28 March 2011.

Foliar C and N concentrations were determined by the combustion of 1-2 mg of pulverized dried sample mixed with 2 mg of V<sub>2</sub>O<sub>5</sub> as oxidant. We coupled the combustion to gas chromatography using a Thermo Electron Gas Chromatograph model NA 2100 (C.E. instruments-Thermo Electron, Milan, Italy). For analyses of other elements (Ca, Fe, K, Mn, P, S), dried and ground samples were digested with concentrated HNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> (30%, p/v) (MERCK, Darmstadt, Germany) in a microwave oven. Measurements were regularly standardized with blank solutions. To assess the accuracy of digestion and the analytical biomass procedures, standard certified biomass (NIST 1573a, leaf tomato, NIST, Gaithersburg, MD) was used. After digestion, the contents of Ca, Fe, K, Mg, Mn, P and S were determined using ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma). By multiplying the elemental concentrations by the aboveground biomass per soil surface area, we obtained the mineralomass of each element, which meant the mass of each element accumulated in biomass per unit of soil surface.

## **Data analysis**

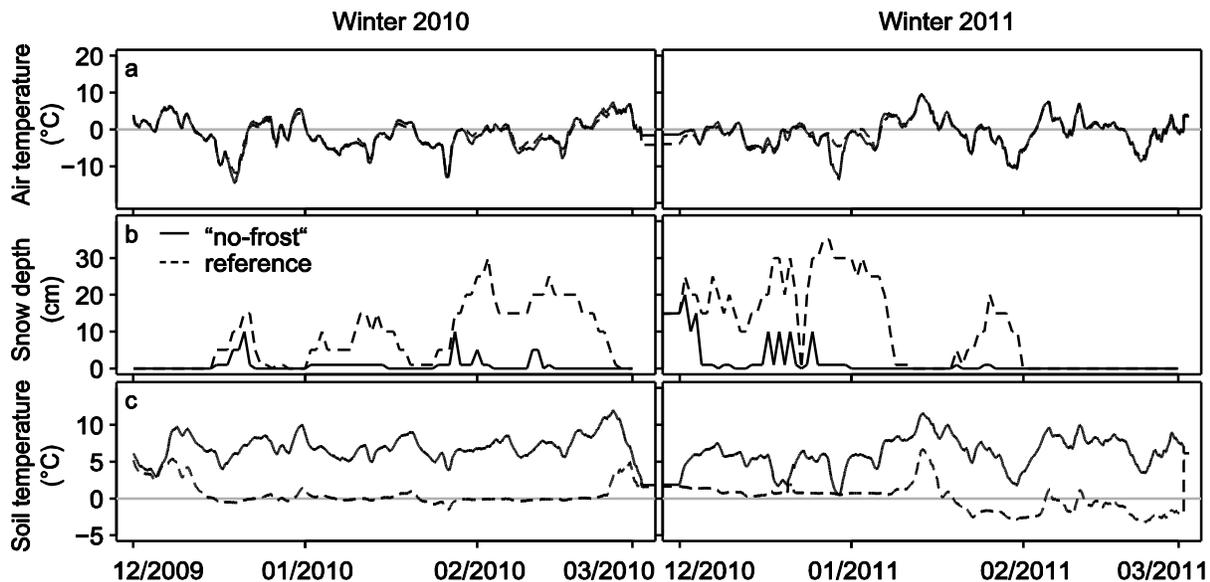
Linear mixed-effect models combined with analysis of variance (ANOVA) were applied to test for significant differences between the “no-frost” and the reference plots. Community composition of the two grassland communities was used as a covariate, after confirming that no

significant interaction between the soil frost manipulations and the community composition occurred. Replication was set as a random factor, thereby accounting for the block design. Before statistical analysis, we tested for normality and homogeneity of variance by examining the residuals versus fitted plots and the normal qq-plots of the linear models (Faraway 2005). If conditions were not met or to improve heterogeneity of variance, data was  $\log(x+1)$ - (soil ammonium and nitrate), square-root- (ANPP) or  $\text{square-root}(x+1)$ - (root length) transformed. Significance level was set to  $p < 0.05$ . All statistical analyses were performed using R 2.12.2 (R Development Core Team 2011) and additional packages *sciplot* (Morales 2011) and *nlme* (Pinheiro et al. 2013). To test for shifts in plant leaf composition after the winter warming treatment we conducted a principal component analyses (PCA). This analysis was performed with all leaf chemical variables (elemental concentrations and their ratios) to analyse differences between treatments and species. Those analyses were performed using Statistica 8.0 (StatSoft Inc., Tulsa, Oklahoma, USA).

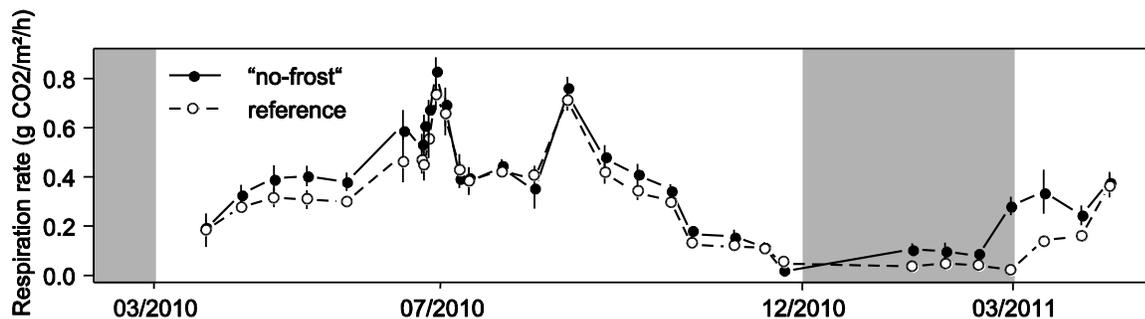
## RESULTS

The soil warming manipulation successfully prevented soil frost during both winters, while the reference plots experienced 46 days of soil frost (32 freeze-thaw cycles) in the first winter and 13 days of soil frost (15 freeze-thaw cycles) in the second winter (Figure 1c). Mean soil temperature in the “no-frost”-plots was higher than in the reference plots by 6.4°C and 6.3°C in the first and second winters respectively. The soil warming led to reduced snow cover in the “no-frost” manipulation (Figure 1b), yet air temperature was not affected by the warming (Fig. 1a).

Soil respiration rate was increased by 291 % (relative difference) in the “no-frost”-treatment during the second winter (1 December 2010 – 28 February 2011) ( $F=7.99$ ,  $P=0.006$ , Fig. 2), but was unaffected outside the warming phase ( $F=2.35$ ,  $P=0.127$ ). The relative increase over the whole observation period (22 March 2010 – 11 April 2011) was 17% (non-significant). Soil nitrogen availability at the upper 10 cm of the soil profile did not differ between the “no-frost”-treatment and the reference plots (ammonium:  $F =0.11$ ,  $P =0.737$ ; nitrate:  $F =2.75$ ,  $P =0.100$ ) (Table 1).



**Figure 1.** Mean daily air temperatures (at +5 cm), snow depth and soil temperatures (at -2 cm) for the “no-frost”-treatment (black line) and reference plots (dotted line) during winter 2009/2010 and winter 2010/2011. Manipulations took place between 1 December 2009 and 1 March 2010 and between 1 December 2010 and 1 March 2011. Mean values over all plots are shown (n=10). Mean values for winter 2010: air temperature -1.3° C, reference soil temperature 0.6° C with 32 FTCs and “no-frost” soil temperature 7.0° C. Mean values for winter 2011: air temperature -0.9° C, reference soil temperature 0° C with 15 FTCs and “no-frost” soil temperature 6.3° C.

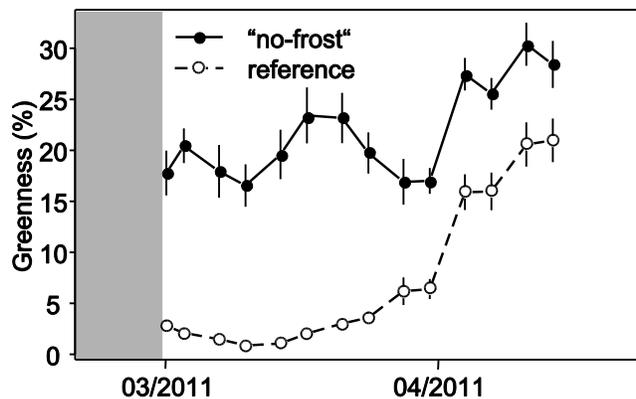


**Figure 2.** Soil respiration for “no-frost”-plots (black dots, solid line) in comparison to reference plots (open dots, dotted line) from 22 March 2010 (after first manipulation) till 11 April 2011. During the “no-frost”-manipulation (grey area) soil respiration was significantly increased ( $F=7.99$ ,  $P=0.006$ ). Mean values and standard error are shown,  $n=5$  for each point

**Table 1.** Mean values of plant-available soil ammonium and nitrate concentrations in the “no-frost” and reference treatment at the different sampling dates (one mixed sample from 0 to -10 cm per plot).

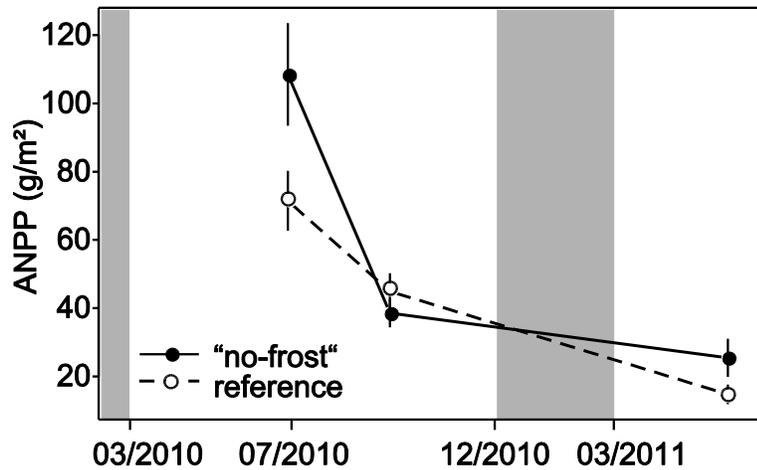
Date	NH4 (mg/l)		NO3 (mg/l)	
	“no-frost”	Reference	“no-frost”	Reference
17.12.09	5.43	6.32.	0.20	0.24
20.01.10	1.98	1.71.	0.15	0.15
18.02.10	1.96	2.02.	0.23	0.25
24.03.10	2.68	2.69.	0.25	0.35
11.03.11	2.50	2.75.	0.16	0.15
23.03.11	2.04	1.49.	0.18	0.18

Plant activity, expressed as aboveground greenness, increased early in the growing season in the “no-frost”-treatment in comparison to the reference plots ( $F=685.71$ ,  $P<0.001$ , Fig. 3). Despite the difference decreasing over time, greenness increased by 195.6% (relative difference) over the observation period.



**Figure 3.** Phenology of greenness for “no-frost”-plots (black dots, solid line) in comparison to reference plots (open dots, dotted line) at the start of the growing season after the second manipulation (grey box) from 1 March 2011 till 14 April 2011. Standardized digital pictures were analysed for their content of green pixels. Highly significant differences in greenness were found ( $F=685.71$ ,  $P<0.001$ ). Mean values and standard errors are shown,  $n=10$  for each point

Total biomass production was increased by 31.5% in the “no-frost”-treatment in comparison to the reference plots ( $F=5.50$ ,  $P=0.024$ ). The effect was greatest after the first manipulation in June 2010 and no longer visible in September 2010. In May 2011, ANPP increased in the “no-frost”-treatment again, but not as much as in June 2010 (Fig. 4).

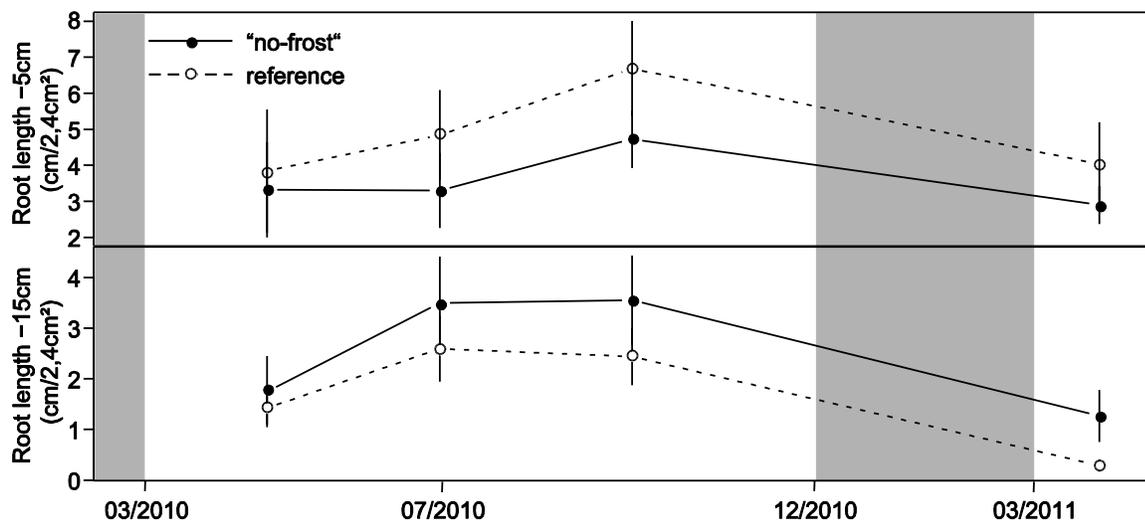


**Figure 4.** Above-ground net primary production (ANPP) over the growing season in 2010 following the first “no-frost”-treatment and in May 2011 after the second “no-frost”-treatment (grey box). Significant differences between the “no-frost”-treatment (black dots, solid line) and reference (open dots, dotted line) were found ( $F=5.50$ ,  $P=0.024$ ). Mean values and standard errors are shown,  $n=10$  for each point

The depth distribution of the roots was affected by the “no frost”-treatment (interaction between treatment and depth:  $F=4.35$ ,  $P=0.039$ ): Root length was reduced at -5 cm depth but increased at -15 cm in the “no-frost”-plots compared to the reference plots (Fig. 5). This translocation of roots had no effect on total root length ( $F=0.03$ ,  $P=0.853$ ).

“No-frost” treatment increased foliar C concentrations ( $F=186$ ,  $P<0.001$  in grasses-only and  $F=115$ ,  $P<0.001$  in grasses&herbs) (Table 2a, b). In the grasses-only community, in addition to having greater plant biomass, the nutrient concentrations were also generally higher in “no-frost” plots than in reference plots. Moreover, in grasses-only plots “no-frost”-treatment increased foliar N ( $F=10.4$ ,  $P=0.007$ ), P ( $F=6.88$ ,  $P=0.022$ ), K ( $F=5.37$ ,  $P=0.039$ ) and S ( $F=7.28$ ,  $P=0.019$ ) concentrations (Table 2a). Contrarily in grasses&herbs community “no-frost” treatment decreased K concentrations ( $F=17.2$ ,  $P<0.001$ ) (Table 2b). The mineralomass of K in “no-frost” treatment was higher than in the reference plots ( $P<0.05$ ) while the mineralomasses of N, P, S and Fe were higher but marginally not significant ( $P<0.1$ ). In grasses-only and in grasses&herbs

communities the PC1 axis separated the scores of the reference samples from those of the “no-frost” treatment (Fig. 6). However, the loading variables were very different in both cases. In grasses-only the PC1 was mainly loaded by larger C, N, P, K and S concentrations in “no-frost” samples whereas in grasses&herbs the PC1 was mainly loaded by the concentrations of K and by its ratios with other elements. Thus, the changes in the elemental concentrations due to the “no-frost” treatment were different in each species when growing in different communities, e.g. in grasses&herbs community, *H. lanatus* plants growing in “no-frost” treatments occupied a PC space towards higher N, P and K concentrations than reference plants, whereas in grasses-only community the contrary was observed (Fig. 6).

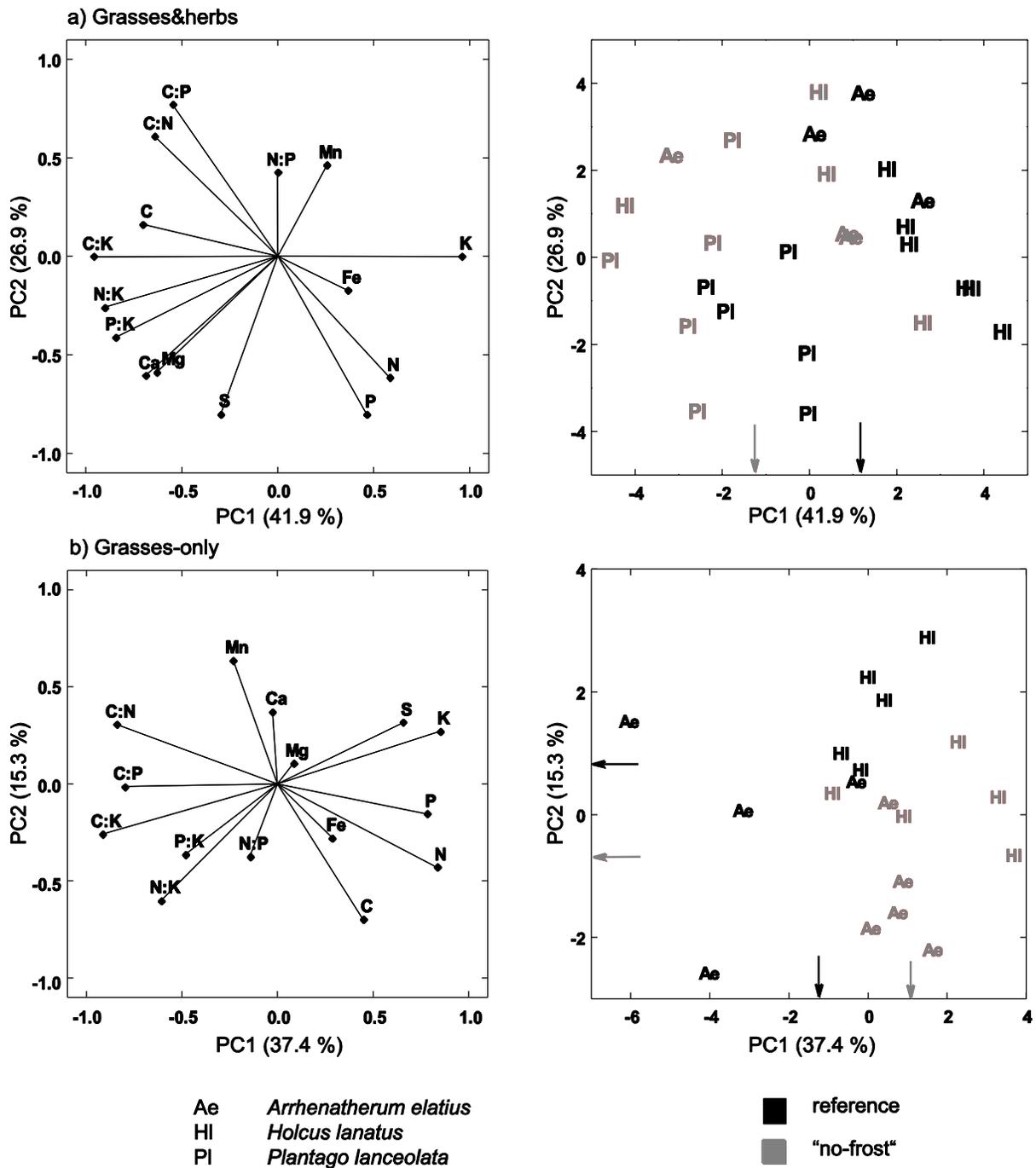


**Figure. 5.** Root length at -5 cm depth and at -15 cm depth measured by minirhizotron technique for “no-frost”-treatment (black dots, solid line) and reference (open dots, dotted line) during the growing season in 2010 and at the start of the growing season in 2011 after the second “no-frost”-treatment (grey box). Total root length did not differ between treatments but root distribution changed (interaction: treatment \* depth,  $F=4.35$ ,  $P=0.039$ ). Mean values and standard errors are shown,  $n=10$  for each point

**Table 2** Mean  $\pm$  S.E. of the elemental concentrations and ratios in different plant species that underwent the “no-frost” treatment or were exposed to reference conditions. (a) Grasses-only community, (b) grasses&herbs community. Different bold letters indicate statistically significant differences ( $P < 0.05$ ). Ae = *Arrhenatherum elatius*. Hl = *Holcus lanatus*. Pl = *Plantago lanceolata*.

<b>Table 2a Grasses-only</b>		<b>Element concentrations (% DW)</b>								
Factor		<b>C</b>	<b>N</b>	<b>P</b>	<b>K</b>	<b>Ca</b>	<b>Mg</b>	<b>S</b>	<b>Fe</b>	<b>Mn</b>
Treatment	Reference	<b>43.1b</b> (0.1)	<b>1.42b</b> (0.06)	<b>0.209b</b> (0.011)	<b>1.11b</b> (0.10)	0.744 (0.056)	0.235 (0.012)	<b>0.187b</b> (0.017)	0.019 (0.003)	<b>0.084a</b> (0.006)
	No-frost	<b>45.2a</b> (0.2)	<b>1.67a</b> (0.05)	<b>0.242a</b> (0.011)	<b>1.37a</b> (0.09)	0.746 (0.048)	0.238 (0.013)	<b>0.218a</b> (0.017)	0.035 (0.008)	<b>0.049b</b> (0.007)
		<b>F=186</b> <b>P&lt;0.001</b>	<b>F=10.4</b> <b>P=0.007</b>	<b>F=6.88</b> <b>P=0.022</b>	<b>F=5.37</b> <b>P=0.039</b>	F=0.000 P=0.99	F=0.0065 P=0.94	<b>F=7.28</b> <b>P=0.019</b>	<i>F=4.01</i> <i>P=0.07</i>	<b>F=14.0</b> <b>P=0.0028</b>
Species	<i>Ae</i>	<b>44.6a</b> (0.4)	1.51 (0.08)	0.218 (0.015)	<b>1.09b</b> (0.08)	0.681 (0.048)	0.230 (0.014)	<b>0.161b</b> (0.009)	0.027 (0.008)	0.055 (0.009)
	<i>Hl</i>	<b>43.8b</b> (0.4)	1.59 (0.06)	0.233 (0.008)	<b>1.39a</b> (0.10)	0.802 (0.047)	0.242 (0.011)	<b>0.241a</b> (0.013)	0.028 (0.007)	0.070 (0.007)
		<b>F=17.8</b> <b>P&lt;0.001</b>	F=1.20 P=0.30	F=1.79 P=0.21	<b>F=6.62</b> <b>P=0.024</b>	F=2.22 P=0.16	F=0.28 P=0.61	<b>F=45.5</b> <b>P&lt;0.0001</b>	F=0.059 P=0.81	<i>F=3.48</i> <i>P=0.09</i>
		<b>Element contents (mg m<sup>-2</sup>)</b>								
		<b>C</b>	<b>N</b>	<b>P</b>	<b>K</b>	<b>Ca</b>	<b>Mg</b>	<b>S</b>	<b>Fe</b>	<b>Mn</b>
Treatment	Reference	385 (90.7)	12.7 (3.0)	1.91 (0.47)	<b>10.7b</b> (2.7)	7.0 (1.7)	2.15 (0.50)	1.76 (0.43)	0.163 (0.041)	0.703 (0.147)
	No-frost	891 (256)	33.4 (9.4)	4.87 (1.44)	<b>26.3a</b> (6.76)	14.2 (4.0)	4.25 (1.16)	4.58 (1.38)	0.492 (0.150)	0.120 (0.435)
		<i>F=3.15</i> <i>P=0.095</i>	<i>F=3.99</i> <i>P=0.060</i>	<i>F=3.49</i> <i>P=0.080</i>	<b>F=4.57</b> <b>P=0.048</b>	F=2.53 P=0.13	F=2.62 P=0.13	<i>F=3.80</i> <i>P=0.069</i>	<i>F=4.49</i> <i>P=0.050</i>	F=1.10 P=0.31
Species	<i>Ae</i>	582 (239)	21.0 (0.9)	3.00 (1.30)	14.7 (6.2)	9.26 (3.65)	2.80 (1.04)	2.37 (1.04)	0.236 (0.076)	0.828 (0.380)
	<i>Hl</i>	714 (197)	26.9 (7.1)	3.89 (1.15)	22.6 (5.5)	12.2 (3.2)	3.67 (0.93)	4.03 (1.21)	0.426 (0.152)	1.09 (0.32)
		F=0.305 P=0.59	F=0.338 P=0.57	F=0.427 P=0.52	F=1.36 P=0.26	F=0.53 P=0.48	F=0.56 P=0.47	F=1.46 P=0.24	F=1.71 P=0.21	F=0.346 P=0.56
		<b>Stoichiometry</b>								
		<b>C:N</b>		<b>C:P</b>		<b>N:P</b>		<b>N:K</b>		<b>P:K</b>
Treatment	Reference	30.8 (1.6)		211 (12)		6.88 (0.31)		41.7 (4.1)		1.35 (0.10)
	No-frost	27.3 (0.8)		190 (8)		6.98 (0.25)		34.1 (2.0)		1.25 (0.07)
		<i>F=4.63</i> <i>P=0.053</i>		<i>F=3.27</i> <i>P=0.096</i>		F=0.006 P=0.94		<i>F=4.04</i> <i>P=0.07</i>		F=0.97 P=0.34
Species	<i>Ae</i>	30.2 (1.7)		212 (14)		7.05 (0.38)		<b>43.0a</b> (3.6)		<b>1.42a</b> (0.08)
	<i>Hl</i>	27.8 (0.8)		190 (6)		6.83 (0.15)		<b>32.9b</b> (2.2)		<b>1.18b</b> (0.07)
		F=2.29 P=0.16		<i>F=3.80</i> <i>P=0.075</i>		F=0.45 P=0.52		<b>F=6.77</b> <b>P=0.023</b>		<i>F=3.51</i> <i>P=0.09</i>

<b>Table 2b Grasses&amp;herbs</b>		<b>Element concentrations (% DW)</b>									
Factor		<b>C</b>	<b>N</b>	<b>P</b>	<b>K</b>	<b>Ca</b>	<b>Mg</b>	<b>S</b>	<b>Fe</b>	<b>Mn</b>	
Treatment	Reference	<b>42.4b</b> (0.1)	1.56 (0.06)	0.261 (0.012)	<b>1.73a</b> (0.15)	1.14 (0.17)	0.255 (0.020)	0.248 (0.019)	0.020 (0.002)	0.035 (0.007)	
	No-frost	<b>44.8a</b> (0.2)	1.44 (0.05)	0.241 (0.010)	<b>1.28b</b> (0.12)	1.31 (0.19)	0.256 (0.023)	0.248 (0.018)	0.029 (0.007)	0.046 (0.008)	
		<b>F=115</b> <b>P&lt;0.001</b>	<i>F=4.0</i> <i>P=0.06</i>	<i>F=3.7</i> <i>P=0.07</i>	<b>F=17.2</b> <b>P&lt;0.001</b>	<i>F=1.24</i> <i>P=0.28</i>	<i>F=0.0001</i> <i>P=0.99</i>	<i>F=0.059</i> <i>P=0.81</i>	<i>F=1.11</i> <i>P=0.31</i>	<i>F=1.02</i> <i>P=0.33</i>	
Species	<i>Pl</i>	<b>44.0a</b> (0.4)	1.47 (0.05)	0.251 (0.014)	<b>1.13c</b> (0.07)	<b>1.91a</b> (0.12)	<b>0.333a</b> (0.018)	<b>0.311a</b> (0.011)	0.018 (0.003)	0.027 (0.01)	
	<i>Hl</i>	<b>43.1b</b> (0.4)	1.56 (0.08)	0.259 (0.013)	<b>1.89a</b> (0.17)	<b>0.883b</b> (0.112)	<b>0.212b</b> (0.010)	<b>0.239b</b> (0.007)	0.035 (0.009)	0.053 (0.038)	
	<i>Ae</i>	<b>43.8ab</b> (0.4)	1.54 (0.06)	0.236 (0.016)	<b>1.49b</b> (0.18)	<b>0.658b</b> (0.040)	<b>0.199b</b> (0.015)	<b>0.161c</b> (0.010)	0.021 (0.005)	0.044 (0.008)	
		<b>F=7.0</b> <b>P&lt;0.01</b>	<i>F=0.93</i> <i>P=0.41</i>	<i>F=0.979</i> <i>P=0.39</i>	<b>F=19.9</b> <b>P&lt;0.001</b>	<b>F=30.2</b> <b>P&lt;0.001</b>	<b>F=24.8</b> <b>P&lt;0.001</b>	<b>F=48.0</b> <b>P&lt;0.001</b>	<i>F=2.95</i> <i>P=0.08</i>	<i>F=3.39</i> <i>P=0.06</i>	
		<b>Element contents (mg m<sup>-2</sup>)</b>									
		<b>C</b>	<b>N</b>	<b>P</b>	<b>K</b>	<b>Ca</b>	<b>Mg</b>	<b>S</b>	<b>Fe</b>	<b>Mn</b>	
Treatment	Reference	656 (128)	24 (4)	3.98 (0.7)	24.5 (4.2)	22.0 (6.4)	4.50 (1.1)	4.2 (0.9)	0.315 (0.067)	0.341 (0.090)	
	No-frost	1000 (380)	31 (1.2)	5.31 (2.2)	24.8 (8.2)	38.0 (19.6)	6.36 (2.62)	6.1 (2.5)	0.707 (0.392)	0.611 (0.229)	
		<i>F=0.930</i> <i>P=0.35</i>	<i>F=0.461</i> <i>P=0.50</i>	<i>F=0.403</i> <i>P=0.53</i>	<i>F=0.002</i> <i>P=0.97</i>	<i>F=0.786</i> <i>P=0.38</i>	<i>F=0.604</i> <i>P=0.45</i>	<i>F=0.660</i> <i>P=0.43</i>	<i>F=0.991</i> <i>P=0.33</i>	<i>F=1.30</i> <i>P=0.27</i>	
Species	<i>Pl</i>	<b>1443a</b> (442)	<b>46.3a</b> (13.5)	<b>8.01a</b> (2.58)	34.2 (8.95)	<b>64.7a</b> (22.9)	<b>10.6a</b> (2.97)	<b>9.78a</b> (2.87)	0.859 (0.491)	0.563 (0.237)	
	<i>Hl</i>	<b>619ab</b> (133)	<b>22.4b</b> (4.8)	<b>3.55b</b> (0.68)	26.5 (5.7)	<b>11.9b</b> (2.6)	<b>3.10b</b> (0.72)	<b>3.31b</b> (0.65)	0.423 (0.126)	0.653 (0.194)	
	<i>Ae</i>	<b>158b</b> (34.4)	<b>5.26c</b> (1.2)	<b>0.849c</b> (0.220)	5.47 (1.37)	<b>2.37c</b> (0.61)	<b>0.711c</b> (0.150)	<b>0.578c</b> (0.146)	0.075 (0.032)	0.036 (0.008)	
		<b>F=4.12</b> <b>P=0.030</b>	<b>F=4.09</b> <b>P=0.031</b>	<b>F=3.71</b> <b>P=0.041</b>	<i>F=3.40</i> <i>P=0.052</i>	<b>F=4.75</b> <b>P=0.019</b>	<b>F=6.08</b> <b>P=0.008</b>	<b>F=5.43</b> <b>P=0.012</b>	<i>F=1.21</i> <i>P=0.32</i>	<i>F=2.14</i> <i>P=0.14</i>	
		<b>Stoichiometry</b>									
		<b>C:N</b>		<b>C:P</b>		<b>N:P</b>		<b>C:K</b>		<b>P:K</b>	
Treatment	Reference	<b>27.6b</b> (1.1)		<b>167b</b> (8)		6.07 (0.23)		<b>30.0a</b> (2.5)		<b>0.964c</b> (0.069)	
	No-frost	<b>31.6a</b> (1.1)		<b>190a</b> (8)		6.04 (0.19)		<b>6.04b</b> (0.19)		<b>1.24a</b> (0.11)	
		<b>F=9.26</b> <b>P=0.007</b>		<b>F=7.63</b> <b>P=0.013</b>		<i>F=0.03</i> <i>P=0.87</i>		<b>F=14.0</b> <b>P=0.0015</b>		<b>F=8.04</b> <b>P=0.011</b>	
Species	<i>Pl</i>	30.2 (1.0)		180 (10)		5.93 (0.20)		<b>40.6a</b> (2.7)		<b>1.34a</b> (0.08)	
	<i>Hl</i>	28.5 (1.9)		171 (10)		6.05 (0.25)		<b>26.0b</b> (4.3)		<b>0.892b</b> (0.098)	
	<i>Ae</i>	30.4 (1.2)		190 (12)		6.26 (0.36)		<b>32.5b</b> (5.3)		<b>1.05ab</b> (0.14)	
		<i>F=0.767</i> <i>P=0.49</i>		<i>F=1.13</i> <i>P=0.35</i>		<i>F=0.496</i> <i>P=0.62</i>		<b>F=8.10</b> <b>P=0.0031</b>		<b>F=9.39</b> <b>P=0.0016</b>	



**Figure 6.** Principal Component Analysis (PCA) conducted with elemental concentrations and ratios as variables and plant samples of different species a) grasses&herbs and b) grasses-only as cases. (*Geranium pratense* was not included because of lack of data due to high mortality). Arrows indicate the mean of sample scores of PC1 axis in controls (black) and in "no frost" (grey) that were significantly different (grasses&herbs:  $F=18.7$ ,  $P=0.0004$ ; grasses-only:  $F=12.8$ ,  $P=0.004$  and  $F=4.95$ ,  $P=0.046$ , respectively)

## DISCUSSION

Soil respiration increased by 291 % in the “no-frost”-manipulation during the second winter. Yet the effect lasted for only two weeks after the warming phase in our study. From then on, we did not observe any difference in comparison to reference conditions. This implies a fast, yet, transient increase in soil biotic activity, which is in line with previous findings (Sharma et al. 2006). The increase in soil respiration in the “no-frost” treatment over the whole observation period is 17% (non-significant). Rustad et al. (2001) found a mean increase of 20% in a meta-analysis of 17 warming experiments with different warming methods and in different biomes. Increased soil biotic activity and soil respiration due to climate warming is viewed as one of the most important positive feedbacks in the climate system (Schlesinger and Andrews 2000). Recently, it has been suggested that such kinds of positive feedbacks were overestimated because water availability will limit soil biotic activity in many systems (Davidson and Janssens 2006; Bontti et al. 2009; Allison and Treseder 2011). Winters in temperate regions, however, do not pose any water limitations on soil biotic activities. In fact, they are projected to become even wetter (Christensen et al. 2007). With ongoing climate warming, winter conditions in the southern temperate zone are expected to reach a point where soil frost gets very rare (Kreyling and Henry 2011), which is in contrast to more northern regions (Henry 2008; Brown and DeGaetano 2011) where decreasing snow cover is projected to lead to more soil frost. It is therefore likely that an acceleration of decomposition is more likely to take place in southern temperate regions than in northern temperate regions.

However, complexities of C dynamics make extrapolation of long-term trends difficult since soil carbon stocks are comprised of strongly contrasting C pools with turnover rates from years to centuries. Understanding the specific responses of different C pools to climate change will be essential for a realistic projection of warming impacts on the carbon cycle (Davidson and Janssens 2006; Conant et al. 2011). It is not certain that increased soil biotic activity and mineralization rates can be sustained by continuous carbon input via primary production (Ineson 1998). Furthermore, species compositions might change because of altered competitive balance in response to winter climate change (Kreyling et al. 2011), and these changes are inherently slow but potentially important for nutrient cycling (Hollister et al. 2005).

Surprisingly, we did not detect increased N availability in the upper 10 cm of the soil during and shortly after the “no-frost”-treatment. Based on increased soil respiration rates, which indicate higher activity of decomposers and N-fixing bacteria, we would have expected increases in decomposition and mineralization, providing more ammonium and nitrate in the soil. We see three logical explanations for this finding: (1) Above-ground greenness of the vegetation early in the growing season suggests increased plant activity already during winter in response to the “no-frost”-treatment. Mineralized nitrogen may therefore have been taken up by the plants in order to fuel their enhanced greenness and growth. (2) Mineralized N may have quickly leached downward in the soil profile in the presence of sufficient moisture with downward flow. (3) Gaseous N could have left the plant-soil system due to increased nitrification or denitrification rates.

The first explanation is based on the fact that plants can maintain photosynthetic activity (Larsen et al. 2007) and N uptake (Grogan et al. 2004; Andresen and Michelsen 2005; Malyshev and Henry 2012) during winter, sometimes comparable to summer N uptake (Nasholm et al. 2000; Bardgett et al. 2003). Unfortunately, we lack samples from deeper soil layers to tackle the second explanation. However, we did observe a shift in rooting depth. Without changes in total root length, significantly more roots occurred in deeper soil layers in the “no-frost”-treatment compared to references, which could be a hint for downward leaching nutrients. Taken together, we see hints supporting both mechanisms. For the third explanation we lack measurements of  $N_2$ ,  $N_2O$  and  $NO$ . Especially in the thawing period after freeze-thaw events there are often gaseous  $N_2O$  and  $NO$  fluxes, but their magnitude differs strongly (Matzner and Borken 2008). In a snow removal experiment, only frozen plots showed  $N_2O$  fluxes whereas unfrozen controls only showed a much smaller flux in spring (Goldberg et al. 2010). Since in our experiment we excluded soil frost, it is not likely that strong  $N_2O$  fluxes occurred. The relative contribution of the three mechanisms, however, is of high ecological importance with regard to nutrient loss and ground water quality and should be investigated in more detail.

The strong increase in aboveground primary production in early summer was not present anymore by autumn. Such stabilisation of ANPP has also been reported in other warming studies (Kardol et al. 2010; Kreyling et al. 2010). During the growing season, depleted nutrient pools could have been the limiting factor for ANPP in “no-frost”-plants. The detected increase in

primary production due to winter warming had to be expected and it agrees with previous findings from temperate ecosystems (Hutchison and Henry 2010), although increased total root length was not supported in our data. Most warming manipulations in high-latitude ecosystems have been conducted during the growing season only (Elmendorf et al. 2012), so we lack studies to compare our results to.

Nutrient composition differed between reference and “no-frost” plants. Most plant nutrients increased their concentrations in “no-frost” plants in spite of higher biomass accumulation, indicating an increased accumulation of nutrients. These results are in accordance with previous reports of higher nutrient accumulation in response to warming in temperate ecosystems (Sardans et al. 2012b). The “Biogeochemical niche” hypothesis proposes that plants competing in the same community use the nutrients in different amounts and proportions, which should diminish the competition for resources among them, such as observed in different Mediterranean plants growing in different climatic conditions (Peñuelas et al. 2008). Plant elemental compositions were affected by the “no-frost” treatment as well as by the plant community composition. In the grasses-only communities, the “no-frost” treatment had a stronger effect on the overall plant elemental concentrations than in the grasses&herbs communities. K concentration in *Holcus lanatus* increased within grasses-only communities but decreased within grasses&herbs communities. The observed shifts in plant elemental composition in response to winter warming deserve further study because stoichiometric changes in plants impact ecosystem trophic webs by favouring herbivores and decomposers with specific nourishment preferences (Sturner and Elser 2002; Sardans et al. 2012a).

Here, we investigated the extreme case of the complete absence of soil frost. Based on climate time series and projections, this is a realistic scenario for Central Europe and large parts of the southern temperate zone (Kreyling and Henry 2011). The mean soil temperature increased by +6.4°C during the “no-frost”-manipulation in comparison to reference conditions, which is at the upper limit of current temperature projections for the end of this century (Christensen et al. 2007). It is important to note that we did not exclude air frost and, consequently direct frost stress to the above-ground parts of the plants. Avoiding air frost completely would represent a highly unrealistic scenario, as temperature fluctuations and minima are projected to occur in the future with persistent magnitudes despite reduced frequencies (Kodra et al. 2011).

During autumn, temperate plants gradually acquire freezing tolerance as temperature and photoperiod decline. The hardening period lasts from days to weeks, dependent on the species and is characterized by increased content of soluble sugars and specific cryoprotective amino acids, as starch content is decreased (Thomashow 1999). Earlier snowmelt (Fig. 1b) and increasing winter/spring temperatures have been shown to advance phenology in many plant species (Ahas et al. 2002; Dunne et al. 2003), even leading to winter growth (Kalberer et al. 2006). Winter growth of plants, indirectly shown by greenness in our data, probably reduces frost hardiness, thereby enhancing the risk of frost damage (Kalberer et al. 2006; Rigby and Porporato 2008; Bokhorst et al. 2009). With vanishing winters plant dormancy can be disrupted altogether, paradoxically causing extended plant dormancy and delayed phenology in spring (Yu et al. 2010). It becomes evident that plant responses to winter warming are complex. To tackle the connected processes and mechanisms in more detail will be an important task in order to identify ecological implications with regard to nutrient leaching or carbon sequestration.

The projected loss of soil frost under future climate conditions over large parts of the temperate zone (Kreyling and Henry 2011) is expected to increase soil respiration, in particular as water availability will not become a limiting factor for biotic activity during winter in these regions. Yet, plant response appears crucial with regard to nutrient leaching and carbon sequestration, as enhanced nutrient uptake and primary productivity may keep nutrient cycles closed (Ineson et al. 1998) and provide additional organic matter to compensate for increased decomposition. Reduced frost hardiness combined with a potential increase in frost damage, however, may counteract this buffering feedback loop and make southern temperate regions prone to increased carbon and nutrient losses in future winters.

## **CONCLUSIONS**

Warmer soils enhanced soil respiration, soil biotic activity, phenology, nutrient accumulation and primary production over winter in our temperate grassland communities. Plant nutrient content and stoichiometry were also altered differently by the absence of soil frost, depending on the species composition of the plant community, indicating that the interaction between climate change and changes in biodiversity is of high ecological importance. In addition,

there was an indication of nutrient leaching (i.e. shifts in rooting depth), which demands quantification in relation to soil nutrient cycles. Furthermore, potential negative feedbacks between winter activity and frost tolerance of the plants require further investigation.

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## Summary

Winter climate change is a complex phenomenon, with snow depth, soil freezing dynamics, and variable air temperature all interacting to bring about differences in among- and within-species growth responses. The objective was to detect growth differences in the responses of species, ecotypes and plant functional groups to winter processes impacted by warmer temperatures. Therefore, experiments were carried out to simulate winter warming and to study its effects on cold acclimation and deacclimation, dormancy loss and frost tolerance. Among-species variation was then compared with within-species variation to determine if a species could be largely treated as a single response unit under different climatic extremes. Plant-soil interactions were also explored to gain a more complete understanding of factors directly impacting plant responses to winter warming. Three in situ experiments, simulating winter warming for different durations and at different amplitudes were conducted for this purpose. Two main questions were posed: (1) what generalities can be found among species- and ecotype-specific plant responses to winter warming simulated under different environmental conditions? (2) what is the role of within species variation in predicting plant responses to climate warming?

Generalities were found among species (relating dormancy depth and its rate of decrease with the passing of winter), within species (latitudinal grass ecotypes showed similar north-south cold acclimation differences as previously shown in trees) and in plant-soil interactions (plant community composition played a major role in N uptake and leaching following prolonged warming and increased temperature variability). This shows that even with high ecotypic and species diversity, experimental biology can provide answers, which apply across species, functional types and experimental conditions. Across several tree species and grass ecotypes the sensitivity to changes in photoperiod was found to influence the effect of temperature on growth cessation and resumption. Photoperiod sensitivity is therefore an important characteristic of plants related to the ability to extend the growing season and resume growth during sudden midwinter warm spells.

With respect to the novel comparison of within-species variation to among-species variation under stress, evidence was found against treating a species as a uniform unit, in terms of its climate change responses, across its distribution. Multiple implications and applications of

high within-species variation are possible. Firstly, the ability of food crop species and species declining in abundance to adapt to warmer temperatures may be improved with assisted migration of better-adapted ecotypes. Secondly, incorporation of within-species variation into models should enable more accurate projections of species distribution changes. Thirdly, preventing extinction and conserving biodiversity can be supplemented by increasing the ecotypic diversity of an area. This way, potentially undesirable side effects from species introductions can be bypassed. For future experiments, this means that the factors which contribute to development of ecotypes should be researched further. Additionally, plant communities with varying degrees of ecotypic and species diversity should be compared in terms of their resilience to climate change impacts.

## Zusammenfassung

Winterklimawandel ist ein komplexes Phänomen, wobei veränderte Schneetiefe, Bodenfrostdynamik und variable Lufttemperaturen miteinander interagieren und zu unterschiedlichen Wachstumsreaktionen führen. Ziel der vorliegenden Arbeit war es, Unterschiede dieser Wachstumsreaktionen auf wärmere Wintertemperaturen in verschiedenen Arten, Ökotypen und funktionellen Pflanzengruppen herauszufinden. Hierfür wurden in Versuchen Wintererwärmung simuliert und die Auswirkung auf Kälteakklimatisierung, De-Akklimatisierung, Dormanzverlust und Frosttoleranz untersucht. Die Variationen zwischen den Arten und innerhalb der Arten wurde miteinander verglichen, um bestimmen zu können, ob eine Art weitgehend als einheitliche Reaktionseinheit unter unterschiedlichen Klimaextremen behandelt werden kann. Die Interaktionen von Pflanzen und Boden wurden ebenfalls untersucht, um ein besseres Verständnis der Faktoren, die die Reaktionen der Pflanzen auf Wintererwärmung beeinflussen, zu erhalten. Zu diesem Zweck wurden drei in situ Experimente durchgeführt, in denen Wintererwärmung in unterschiedlichen Zeiträumen und Amplituden simuliert wurde. Zwei zentrale Fragen wurden aufgestellt: (1) Welche allgemeingültigen Regeln können für pflanzenart- und ökotypspezifische Reaktionen auf Wintererwärmung unter unterschiedlichen Umweltbedingungen abgeleitet werden? (2) welche Rolle spielen Variationen innerhalb der Arten bei der Vorhersage von Pflanzenreaktionen auf den Klimawandel?

Solche allgemeingültigen Regeln konnten abgeleitet werden. So wurde im Vergleich der Arten untereinander eine enge Abhängigkeit zwischen der Dormanztiefe und dem Dormanzverlust zum Ende des Winters festgestellt. Innerhalb der Arten fanden sich für Grasökotypen ähnliche Breitengradabhängige Unterschiede in der Kälteakklimatisierung, wie sie für Bäume bereits bekannt waren. Bezüglich der Pflanze-Boden-Interaktionen konnte nachgewiesen werden, dass die Zusammensetzung der Pflanzengemeinschaft eine entscheidende Rolle bei der Stickstoffaufnahme und -auswaschung nach längeren winterlichen Wärmephasen und erhöhter Temperaturvariabilität spielt. Diese Beispiele zeigen, dass trotz ökotypischer und zwischenartlicher Variabilität die experimentelle Ökologie Antworten geben kann, die generell über die einzelnen Arten, funktionelle Typen und Versuchsstandorte hinaus gelten. Darüber hinaus stellte sich heraus, dass die Photoperiode ebenfalls eine wichtige Rolle für die Beendigung sowie für die Wiederaufnahme des Wachstums bei einigen

Baumarten und Grasökotypen spielt. Die Sensitivität der Pflanzen für die Tagelänge in Hinblick auf ihre Dormanz ist deshalb ein wichtiges Merkmal der Pflanzen in Bezug auf ihre Fähigkeit, bei plötzlich einsetzenden Wärmeperioden im Winter und frühere warme Temperatur in der Frühling die Vegetationsperiode zu verlängern.

In Bezug auf den neuartigen Vergleich von innerartlicher und zwischenartlicher Variabilität unter Stress zeigen die Ergebnisse, dass die Behandlung einer Art als eine einheitliche Einheit über ihr gesamtes Verbreitungsgebiet in Bezug auf ihre Reaktionen gegenüber dem Klimawandel zu kurz greift. Aus der hohen innerartliche Variation ergeben sich mehrere Schlussfolgerungen und Anwendungen. Erstens, die Anpassungsfähigkeit von Nutzpflanzen oder rückläufigen Arten an wärmeren Temperaturen kann verbessert werden durch unterstützte Migration von besser angepassten Ökotypen. Zweitens, der Einbau von innerartlicher Variabilität in Artverbreitungsmodelle wird genauere Prognosen für zukünftige Arealveränderungen ermöglichen. Drittens, die Bemühungen zur Vermeidung des Aussterbens von Pflanzenarten und zum Erhalt einer hohen Biodiversität können durch eine Erhöhung der ökotypischen Vielfalt in einem Gebiet unterstützt werden. Auf diese Weise können potentiell unerwünschte Nebenwirkungen von Arteneinführungen vermieden werden. Die Entwicklung von Ökotypen und ihre Faktoren bedürfen darum weiterer wissenschaftlicher Untersuchungen. Darüber hinaus sollten Pflanzengemeinschaften mit unterschiedlicher Diversität der Arten und Ökotypen in ihrer Widerstandsfähigkeit gegenüber den Auswirkungen des Klimawandels verglichen werden.

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