Cold acclimation in warmer extended autumns impairs freezing tolerance of freezing tolerance of perennial ryegrass (*Lolium perenne* L.) and timothy (*Phleum pratense* L.)

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The effect of variable autumn temperatures in combination with decreasing irradiance and daylength on photosynthesis, growth cessation and freezing tolerance was investigated in northern- and southern-adapted populations of perennial ryegrass (*Lolium perenne* L.) and timothy (*Phleum pratense* L.) intended for use in regions at northern high latitudes. Plants were subjected to three different acclimation temperatures; 12, 6 and 9/3˚C (day/night) for four weeks, followed by one week of cold acclimation at 2˚C under natural light conditions. This experimental setup was repeated at three different periods during autumn with decreasing sums of irradiance and daylengths. Photoacclimation, leaf elongation and freezing tolerance were studied. The results showed that plants cold acclimated during the period with lowest irradiance and shortest day had lowest freezing tolerance, lowest photosynthetic activity, longest leaves and least biomass production. Higher acclimation temperature (12˚C) resulted in lower freezing tolerance, lower photosynthetic activity, faster leaf elongation rate and higher biomass compared to the other temperatures. Photochemical mechanisms were predominant in photoacclimation. The northern-adapted populations had a better freezing tolerance than the southern-adapted except when grown during the late autumn period and at the highest temperature; then there were no differences between the populations. Our results indicate that the projected climate change in the north may reduce freezing tolerance in grasses as acclimation will take place at higher temperatures and shorter daylengths with lower irradiance.

Key words: Cold acclimation, chlorophyll fluorescence, freezing tolerance, leaf elongation, geographically adapted populations, climate change

Abbreviations - F_m (F'_m), maximal chlorophyll fluorescence yield in the dark-adapted (light-adapted) leaf; F_o (F'_o), minimum chlorophyll fluorescence yield in the dark-adapted (light-adapted) leaf; F_s, steady-state chlorophyll fluorescence yield in the light-adapted leaf; F_v, F_m-F_o; $\phi_{PSII,}$ current quantum yield of PSII; q_p, coefficient of the photochemical quenching of chlorophyll fluorescence; NPQ, non-photochemical quenching of chlorophyll fluorescence.

Introduction

Autumn and winter temperatures are predicted to increase considerably at higher northern latitudes the coming decades (ICPP 2013). This in combination with the low irradiance and short day length at these latitudes can intensify or give new problems with overwintering of perennial forage crops. Rising winter temperatures and fluctuating weather conditions with unstable snow cover could increase the winter stresses of plants (Bertrand and Castonguay 2003, Uleberg et al. 2014, Cooper 2014). The predicted changes in autumn climate may affect cold acclimation of perennial plants negatively.

Cold acclimation of herbaceous species has been widely studied (reviewed by e.g. Thomashow 1999, Cinnusamy et al. 2006, Sandve et al. 2011, Quellet and Charron 2013, Wingler 2015) and is a process where climatically adaptive plants can increase their freezing tolerance in response to low non-freezing temperatures. Freezing tolerance is a dynamic character affected by environmental factors such as temperature and light (Gray et al. 1997) and is both seasonally (Yoshida et al. 1997, Palva et al. 2002) and diurnally (Keily et al. 2013) controlled. A decrease in temperature during late summer/early autumn triggers changes in the gene expression, resulting in increased freezing tolerance of the plant (Cinnusamy et al. 2006). In the plant cell, the chloroplast may be the primary site for cold sensors of ambient temperatures in addition to the plasma membrane (Miura and Furumoto 2013). As reviewed by Hüner et al. (2014), the formation of an excitation pressure within photosystem II (PS II) in photosynthetic active tissue as a response to decreasing temperatures is the sensor for cold regulated mechanisms; not the low temperature per se. Excitation pressure develops as a response to over-reduction of PS II since electron transfer through the electron transport chain is too slow (Hüner et al. 2013). This situation occurs either as a response to low temperature, which reduces the rate of carbon assimilation and hence the need for photochemical energy; or as a response to high light conditions (Ensminger et al. 2006). This redox sensing signalling through excitation pressure is both species and cultivar dependent (Hüner et al. 2013). Adaptive genotypes can avoid photoinhibition and start a process of photoacclimation either by increasing the rate of energy dissipation by non-photochemical quenching mechanisms (NPQ) or by enhancing the rate of carbon assimilation and photosynthetic performance through a process of photochemical quenching (q_p) (Hüner et al. 2012). As a result, photoacclimated plants exhibit a higher maximum photochemical efficiency (Fv/Fm) and increased photosynthetic activity (ϕ_{PSII}) compared to non-acclimated plants. The capacity of the plant to photoacclimate correlates with freezing tolerance (Hüner et al. 1993, Rapacz et al. 2004) and tolerance to high light intensities (Rapacz et al. 2008).

In woody species, cold acclimation is a two-step process controlled by a combination of short photoperiod and low temperature, where growth cessation is followed by cold acclimation (Junttila 1996). In grasses and herbaceous species, data on impacts of photoperiod on cold hardening are still scarce. Although temperature seems to be the main factor, cold acclimation of grasses is also triggered

by photoperiod, especially at higher temperatures (Malyshev et al. 2014). Likewise, hardening of white clover (*Trifolium repens* L.) is enhanced by short photoperiod (Junttila et al. 1990). Recent studies show that the C-repeat binding factor (CBF) cold acclimation pathway in *Arabidopsis thaliana* is regulated by photoperiod (Lee and Thomashow 2012). At higher temperatures, long days caused repression of the CBF pathway, while short days relieved the repression resulting in increased freezing tolerance. This indicates that cold acclimation of herbaceous species is not only controlled by a decreasing temperature, but also photoperiod. Cold acclimation of grasses is also affected by the light intensity (Pollock et al. 1988, Harrison et al. 1997, Höglind et al. 2010), light quality and length of the hardening period (Sjøseth 1964). Winter-hardy cultivars of grasses of northern origin start hardening earlier and achieve a higher freezing tolerance than southern-adapted, less winter-hardy cultivars (Larsen 1994). Longer days stimulate dry matter production in perennial grasses (Hay 1990), and the growth of cultivars adapted to higher northern latitudes are most sensitive to photoperiod (Heide 1982, Solhaug 1991). However, very few studies have focused on temperature × light interactions during autumn at northern high latitudes. In order to achieve full hardening, active growth in the plants must cease (Rapacz 1998a). The mechanism behind growth cessation of grasses is still poorly understood (Rapacz et al. 2014), but northern-adapted forage grasses seem to have a specific mechanism for growth inhibition during autumn (Østrem et al. 2014).

The light regime at northern high latitudes is profoundly different from light regimes at temperate or tropical latitudes (Nilsen 1985). In autumn, the daylength and the global irradiance decrease rapidly with modifying effects of clouds (Fig. 1). The light quality is unique at higher latitudes, with less diurnal alterations, but higher annual variation in the ratio of red and far red light compared to lower latitudes (Nilsen 1985). According to future climate projections, the onset of low positive temperatures required for cold acclimation (<10°C) will occur later in the autumn and under considerably shorter daylength and lower irradiance than today (IPCC 2013). It is unknown how higher temperature in combination with reduced irradiance and shorter day length will affect cold acclimation and freezing tolerance of plants in future climate.

Timothy (*Phleum pratense* L.) is the most common forage grass species in Northern Norway due to its superior winter survival and good growth at low temperatures and long days. However, when the growing seasons extend into late autumn due to climate changes, perennial ryegrass (*Lolium perenne* L.) may be better adapted in the north than currently. The purpose of this study was to examine the physiological responses of these grasses to a warmer future autumn climate, in order to understand which traits will be important for breeding cultivars for future climatic conditions at higher northern latitudes.

We compared photoacclimation, growth rates and freezing tolerance of promising breeding populations of perennial ryegrass and timothy under different combinations of autumn light and temperature in a phytotron at Holt, Tromsø, Norway (69.68°N, 18.94°E). In an earlier study (Dalmannsdottir et al. 2016), we tested the effect of different pre-acclimation temperature treatments

on freezing tolerance of the same grass populations and red clover (*Trifolium pratense* L.). In the current experiment, we exposed plants to three successive periods of natural light during autumn, creating three distinct light regimes of progressively lowered irradiance and daylength (Fig. 1). Our hypotheses were; (1) hardening under natural light conditions later in the autumn, at shorter daylength and lower irradiance, reduces the freezing tolerance of perennial ryegrass and timothy; (2) high temperature (12°C) reduces hardening compared to low temperature (6°C), and variable day-time and night-time temperatures (9/3°C), compared with a constant temperature (6°C) affect hardening differently because of diurnal effects; (3) the level of freezing tolerance is regulated by an interaction between temperature and irradiance/daylength; and (4) northern-adapted populations are more sensitive than southern-adapted populations to changing light and temperature conditions.

Materials and Methods

Plant material and growth conditions

Two forage grass species, perennial ryegrass and timothy, were studied. Two populations of each species were included, one selected for the northern regions of Norway (northern-adapted) and the other for the southern regions of Norway (southern-adapted). The perennial ryegrass populations were FuRa9805 (southern-adapted, lat. 55°N (original material) and 61°N (natural selection)) and Fagerlin (northern-adapted, lat. 55°N-63°N, adapted to low winter temperatures), and the timothy populations MTL9701+Grindstad (southern-adapted, lat. 50°N-60°N) and MTV0508-3 (northern-adapted, selected at lat. 59°N/500 m a.s.l. (one generation) and at 67°N (two generations)). For detailed description of populations see Dalmannsdottir et al. (2016).

The experiment was conducted in autumn 2012 at Holt, Tromsø (69.68°N, 18.94°E) in phytotron compartments with the temperature controlled to ±0.5°C and the air humidity corresponding to a water vapour deficit of 0.5 kPa. Seedlings were planted in tree nursery trays (60 pots in each tray, one plant/pot, pot size 40 mm diameter x 85 mm height, 45 mm spacing between plants). The pots were filled with fertilised sphagnum peat and perlite (3:1). The plants were watered regularly and fertilised as required with a complete nutrient solution (Hoagland solution, modified from Asher 1978). Apart from the establishment phase, light conditions were natural light during the whole experiment. During establishment, the plants were grown under controlled light conditions in light-isolated chambers for 4 weeks at 20°C, 24 h photoperiod. The light source was cool white fluorescent lamps (Philips TLD 58W 840), giving 150 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at plant level (measured with a quantum sensor, Li-1000, Li-Cor) within the range 400-750 nm. During the experimental periods, plants were placed in glasshouse phytotron chambers, allowing natural light from all sides.

Experimental design

The whole experiment was conducted under natural light conditions in a phytotron during three separate periods in autumn (year 2012); 5 Sept-10 Oct (early period), 26 Sept -31 Oct (intermediate

period) and 17 Oct-21 Nov (late period), resulting in three irradiance/day length treatments here referred to as early, intermediate and late autumn period (Fig. 1). The day length decreased approximately from 14 to 9 h (early period), 11 to 6 h (intermediate period) and 8 to 2 h (late period) during the three periods. The sum of global irradiances for each period decreased from 154, 76 to 21 W m^{-2}, respectively. After establishment, similar sized plants were selected for the experiment. Each population was planted in separate trays, which were placed randomly on trolleys within the phytotron compartments. To ensure that plants were at a similar stage and phenology (4 weeks old seedlings) when entering the experiment, we established new seedlings for each successive period. Hence, they were sown at three different dates (8 August, 29 August, 19 September) (Fig. 2).

After establishment, plants were subjected to three pre-acclimation temperature treatments, 6, 9/3 (12 /12 h) and 12°C, in combination with the three autumn periods (early, intermediate, late) (Fig. 2). The duration of the pre-acclimation temperature treatments was 4 weeks, and thereafter all populations were cold acclimated at 2°C for one week before freezing tests were conducted. The 6°C treatment resembles the current temperature in Sept-Oct in Northern Norway and 12°C an extreme temperature increase in the autumn based on future scenarios until 2050 (Uleberg et al. 2014). We used 6°C and 9/3° daytime 08:00 to 20:00/night-time 20:00 to 08:(12h/12h) treatments, giving the same daily temperature sum, to study the influence of changing day and night temperatures on pre-acclimation efficiency and subsequent freezing tolerance levels. Temperature loggers inside the growth chambers secured the accuracy of temperature measurements, but measurements of water vapour deficit were more unstable for the lower temperatures (2 and 6°C), often 30-40% higher than programmed.

Morphological measurements

Dry weight of aboveground biomass of 15 plants per population per treatment was recorded at the start of the temperature treatment and at the end of the experiment (Fig. 2), after drying at 60°C for 48 h. The aboveground biomass produced during the experiment was obtained by calculating the difference between the measurements at the start and at the end. Leaf elongation (mm $week^{-1}$) was measured on 15 plants per treatment (in total 180 individuals per autumn period). The youngest emerging leaf on each plant was marked with a thin rubber band and measured weekly during the 5 weeks of pre- and cold acclimation treatments.

Chlorophyll fluorescence measurements

The photochemical activity of photosystem II (PSII) was studied by measuring chlorophyll fluorescence (PAM-2500 Portable Chlorophyll Fluorometer; Heinz Walz, Effeltrich, Germany) at room temperature on 15 plants per treatment before and after pre-acclimation treatment, as well as after cold acclimation (Fig. 2) (totally 810 plants for each autumn period), on the same plants as were used to record leaf elongation. The measurements were made on the mid-section of the youngest fully expanded leaves. Before measuring maximum quantum yield of PSII (F_v/F_m) (indication of photoinhibition), leaves were

dark-adapted for 15-60 min in leaf clips (8 mm diameter, Walz) and values of F'_m and F_s were recorded when F_s became stable after re-exposure to actinic red light (800 µmol). Within the same leaf clip, F'_0 was measured after far-red light treatment to ensure rapid opening of PSII reaction centres. Current quantum yield of PSII (ϕ_{PSII}) (photosynthetic activity indicator) and coefficients of the photochemical (q_p) and non-photochemical (NPQ) quenching of chlorophyll fluorescence were calculated according to Genty et al. (1989), Schreiber et al. (1994) and Bilger and Björkman (1991), respectively.

Freezing test

At the end of the experiment, freezing tests were performed as described by Pulli et al. (1996) with modifications (Höglind et al. 2010) (Fig. 2). Plant roots were washed and single plants were trimmed to 3 cm top and 1-2 cm root. The crown segments were placed in plastic boxes and covered with fine, humid sand in a programmable freezer with a temperature sensor in each box. Before freezing treatments commenced, the temperature was lowered from 2°C to -3°C by 1°C h^{-1} and kept at -3°C for 13 hours to avoid super-cooling of the plants. The boxes were then frozen to pre-determined temperatures between -3 and -20°C with 2°C intervals, depending on species and treatment. Freezing progressed at a cooling rate of -1°C h^{-1} until -10°C was reached; from then on, the cooling rate was -3°C h^{-1} until the predetermined temperature was reached for each treatment. There were two replicate boxes per predetermined test temperature; each containing 10 crown segments per population per treatment, i.e. 480 plants per temperature treatment and autumn period summing up to a total number of 4320 plants. Two boxes per population per treatment were kept at 2°C in darkness as a control. After freezing, the boxes were placed at 2°C in the dark overnight to thaw, and the crown segments were transplanted into fertilised peat mixed with perlite. Survival of individual plants was rated as dead or alive and the LT_{50} value, i.e. the temperature at which 50% of plants are killed, was estimated after 3-4 weeks at 20°C and 24 h light (approximately 150µmol m^{-2} s^{-1}).

Statistical analysis

A generalised linear model approach was used to estimate the effects of pre-acclimation temperature on photosynthetic activity, biomass production and freezing tolerance. Model selection was based on the Akaike's Information Criteria corrected for small sample sizes (AICc) (Burnham and Anderson, 2002), as this approach is more robust when evaluating interaction effects (Crawley 2007, Gelman and Hill 2008). For biomass production and photosynthetic activity, a linear model with Gaussian normal distribution and an identity link was assumed. The full model was defined as *Response ~ Treatment*Population*Species*autumn period,* where temperature treatment, population and autumn period are treated as categorical variables. Four entries were removed as diagnostic plots indicated them as outliers. Data was log-transformed when variance was heterogeneous (Kleinbaum et al. 1998), however, untransformed mean values are presented here for clarity. For the freezing test, a logistic model with logit link function was used. In some of the models there was no overlap of freezing

temperature for the group of dead and surviving individuals, hence penalized likelihood was used to remove bias (library brglm in R) (Kosmidis and Firth 2009). The full model for each species was defined as *Response ~ Treatment*Population*Pre-determined freezing temperature.* We used a non-linear, three-parameter asymptotic mixed model for estimating leaf elongation (function nlmer in the R library lme4). The growth trajectory is described by the function $Leaf\ length \sim Asym + (R_0\text{-}Asym)e^{-e(lrc \times week)}$, where parameters describe the intercept (R_0), the asymptote (*Asym*) and the logistic rate constant (*lrc*) (Crawley 2007). Here, the R_0-value describes leaf length at the start of the experiment and the *Asym* value describes the leaf length at cessation of growth. While the lrc-value describes the logistic growth rate constant, the absolute growth rate at a certain time is given by the combination of the parameters as described by the non-linear function above. Individual plant identity was included as a random term to avoid pseudoreplication. As we were interested in main effects and the effects sizes, we chose to evaluate differences in leaf elongation by comparing 95% confidence intervals rather than multiple comparison approaches (Saville 2015, Garcia 2004, Rothman 1990). For non-linear mixed models, the confidence interval was approximated by mean ± 2 x SE (Gelman and Hill 2008). Predictors were considered significant if their 95% confidence interval did not include zero. All statistical analyses were performed using R (R version 3.0.1) and Minitab 16 (Minitab Inc. 2010, State College, PA, USA). Model comparisons and population statistics are presented in supplementary table S2-15.

Results

Biomass production

Total biomass production decreased gradually from the early autumn period to the late period. However, the decrease was also dependent on temperature (Table 1). During the early autumn period, markedly more biomass was produced at 12 compared to 6 and 9/3°C (Table 1). Southern-adapted populations also produced more biomass at 9/3 compared to 6°C during this period. During the intermediate autumn period, plants at 12 and 9/3°C produced more biomass than plants at 6°C, though not significant for the northern-adapted population of perennial ryegrass (Table 1). During the late autumn period, there were no significant differences in biomass production between temperature treatments (Table S3). We found no consistent differences in biomass production between northern and southern-adapted populations (Table S3). However, in particular northern-adapted perennial ryegrass produced more biomass than timothy at 6°C and during the early autumn period.

Leaf elongation

The effect of autumn period on leaf elongation was dependent on the temperature treatment and population. Leaf elongation rate was always higher at 12 than at 6 and 9/3°C for all autumn periods and populations (reflected in higher lrc value, Fig. 3, Table S1). The effect of temperature on leaf elongation was more pronounced at the late autumn period in particular for northern perennial ryegrass

(Fig. 3, Table S1). Leaf elongation of plants at 12°C was faster at later autumn periods compared to earlier periods (Fig. 3, Table S1), though not significantly different for the southern-adapted population of perennial ryegrass at the early and intermediate autumn periods. In the late autumn period, leaves ceased growth at lower leaf length at 6°C than at 12 or 9/3°C resulting in longer leaves at 12 and 9/3°C (reflected in lower asymptote values of leaf length at 6°C, Table S1). Only the southern-adapted population of perennial ryegrass showed no difference in elongation rate between treatments at 6 and 9/3°C in the early period. During the intermediate autumn period, all populations grew significantly faster at 9/3°C compared to 6°C. In the late autumn period, the same effect was observed in perennial ryegrass, but was not significant in the timothy populations. There was no general difference between southern and northern-adapted populations regarding leaf elongation rate.

Chlorophyll fluorescence

The photochemical activity measured after pre-acclimation temperature treatments is presented in Fig. 4. The photochemical efficiency ($F_v/F_{m)}$ were overall higher in the intermediate autumn period than in the early and late autumn periods (Fig. 4, Table S8). In the early autumn period, $F_v/F_{m,}$ increased at higher temperatures, except for plants of the southern-adapted timothy population (Fig. 4, Table S8). Opposite, in the late autumn period, F_v/F_m values decreased at increasing temperatures. The non-photochemical quenching (NPQ) values were highest in the intermediate autumn period for both species. In timothy, plants at 12°C had the lowest and plants at 6°C the highest NPQ values at both late and intermediate autumn periods (Fig. 4, Table S9), whereas this was not evident in the early autumn period. This temperature effect was not observed in the northern-adapted and only in the late autumn period in the southern-adapted perennial ryegrass.

Photosynthetic activity (ϕ_{PSII}) was affected mainly by autumn period; it decreased with later autumn periods and increasing temperature (Fig. 4, Table S10). Photochemical quenching (q_p) showed similar trends as ϕ_{PSII} (Fig. 4, Table S11). Photochemical activity before pre-acclimation treatments was not significantly different between populations and treatments, with the exception of F_v/F_m which varied slightly but not consistently between populations and treatments (data not shown). After pre-acclimation, F_v/F_m and NPQ values increased for all temperature treatments and autumn periods compared to before pre-acclimation, as observed by Dalmannsdottir et al. (2016). Also, q_p and ϕ_{PSII} values increased after pre-acclimation, except at the late autumn period when values before pre-acclimation were higher. The only change in photochemical activity after cold acclimation at 2°C was a slight increase in F_v/F_m values at the early autumn period, especially at lower temperature (data not shown).

No significant differences were found between northern- and southern-adapted populations regarding photochemical activity.

Freezing tolerance

Plants pre-acclimated in the late autumn period and at the highest temperature displayed lowest freezing tolerance irrespective of species and population (Table 2, Fig. 5). Plants pre-acclimated at 12ºC were less freezing tolerant than plants acclimated at 6 and 9/3ºC. There were no significant differences in freezing tolerance between the 6 and 9/3ºC treatments (Table S14, S15). There was no significant differences between plants pre-acclimated in the early and the intermediate autumn period. Northern-adapted populations had higher freezing tolerance compared to southern ones, except for those pre-acclimated in the late autumn period and at the highest acclimation temperature (Fig. 5, Table 2). In the intermediate and the late autumn period freezing tolerance was more strongly reduced by the 12 than the 6°C temperature treatment in northern-adapted populations compared to southern-adapted (Fig. 5, Table 2).

Discussion

We found that interactions between temperature and day length/irradiance had strong effects on growth, cold acclimation and freezing tolerance of perennial ryegrass and timothy populations with diverse adaptations. A combination of low irradiance/short day length and higher than normal temperatures, a scenario expected with global warming at higher latitudes, reduced freezing tolerance and photosynthetic activity substantially in all populations. The northern-adapted populations generally had higher freezing tolerance than the southern-adapted, but not at the combination of shortest day length and highest temperature. This indicates that populations adapted at higher latitudes are vulnerable to the predicted climate changes, which will be most pronounced in these regions.

Temperature and daylength/irradiance effects on growth

All populations responded to a lower irradiance and shorter daylength with reduced dry-matter production. This is in accordance with previous studies of timothy and other high latitude grass species (Heide et al. 1985, Solhaug 1991, Wu et al. 2004). In our study, leaf elongation rate was similar (at 6°C) or increased (9/3 and 12°C) at later autumn periods. At later autumn periods, the plants (especially timothy) were suffering from low turgor pressure despite normal soil humidity (data not shown). Leaves of timothy were thin and etiolated, while leaves of perennial ryegrass were narrow but with more turgor than timothy, especially plants of northern-adapted perennial ryegrass. Etiolated growth of grasses is a well-known response to limited light conditions (Robson et al. 1988). Peri et al. (2007) also found that etiolated pastures with cocksfoot produced less dry matter. Schnyder and Nelson (1988) found that leaf elongation in tall fescue (*Festuca arundinacea*) was up to 65% faster during the dark period during the diurnal cycle, depending on the light intensity and temperature shift. Leaf elongation in grasses is known to increase at higher temperatures, and this was confirmed in the present study and in a previous study where the same populations were tested for temperature responses (Dalmannsdottir et al. 2016). Different day/night temperature (9/3°C) stimulated biomass

production and leaf elongation compared to the corresponding constant temperature (6°C) in the early and intermediate autumn period, especially in southern-adapted populations. During the late autumn period, the light level was a limiting factor, thus there were no temperature effects on biomass production. However, leaf elongation was stimulated at 9/3 but not at 6°C, as a response to lower irradiance and shorter dayl ength. Junttila (1985) found that shoot elongation of timothy cultivars was stimulated by alternating temperatures compared to corresponding constant temperatures, possibly related to light × temperature interactions. The same effect has been shown in pea (*Pisum sativum*) (Grindal et al. 1998) and oilseed rape (*Brassica napus* L. *var. oleifera*) (Rapacz 1998b).

Under low temperature and long day conditions, it has been shown that dry matter production is generally more strongly stimulated in grass cultivars adapted to northern high latitudes compared to ecotypes from lower latitudes (Solhaug 1991, Østgård and Eagles 1971). Our results did not show significant differences in biomass production between northern and southern-adapted populations, except for the northern-adapted population of perennial ryegrass (cv. 'Fagerlin'), which produced more dry matter at 6°C and during the early autumn period compared to the southern-adapted. Furthermore, at the late autumn period, we observed that the northern-adapted populations of both species had a more compact growth habit than the southern-adapted. The northern-adapted perennial ryegrass, cv. 'Fagerlin', showed relatively high photosynthetic activity and reduced leaf elongation growth during autumn in a field study in Norway (Østrem et al. 2014). It had good winter survival and high plot coverage the following spring. Together with our findings this indicates that cv. 'Fagerlin' may be able to utilize a prolonged growth season without sacrificing the level of freezing tolerance. This cultivar may thus be a promising germplasm resource for future breeding programs.

Temperature and daylength effects on photoacclimation

Cold acclimation is known to increase photosynthetic performances (Yamasaki et al. 2002; Hüner et al. 2014), which results in higher PSII photosynthetic activity at lower temperatures (Dalmannsdottir et al. 2016). This is supported by our study especially under early autumn light conditions. Photosynthetic activity was more affected by autumn period than temperature, and light conditions during later autumn periods reduced the photosynthetic activity in all populations. Increasing q_p (photochemical quenching) with increasing irradiance/day length and decreasing temperature shows that the photochemical acclimation mechanism was more predominant than the non-photochemical mechanism, as demonstrated before in a response to temperature in studies with winter rye (Huner 1985) and oilseed rape (Rapacz and Janowiak 1998). Our results did not indicate an active NPQ mechanism, but the NPQ values observed in timothy at the two later autumn periods may be caused by etiolation of leaves rather than temperature. In etiolated leaves and leaves at low light intensities, the amount of active PSII reaction centres is reduced (Miyata et al. 2012) as an adaptive response to

protect the photosystem (Tikkanen et al. 2014), resulting in lower NPQ values. The higher sensitivity of timothy compared to perennial ryegrass in relation to leaf etiolation is reflected in the fluorescence measurements. NPQ mechanisms have been found to dissipate excess light during cold acclimation in winter hardy grass species (Humphreys et al. 2007) and northern-adapted cultivars (Rapacz et al. 2004). In our study, southern and northern-adapted populations were not different as regards q_p mechanisms. A slightly higher ϕ_{PSII} at 6 compared to 9/3°C indicates lower excitation pressure in plants at 6°C because of lower temperature during the daylight period. Photoinhibition was observed at the early autumn period in combination with low temperature. A shift from the early autumn period (5 Sept -10 Oct) to the intermediate (26 Sept - 31 Oct) reduced the damages of PSII, expressed as higher F_v/F_m values. However, reduction of the autumn light conditions during the late autumn period decreased the Fv/Fm values again, probably because the irradiance was below a critical limit for the induction of photoacclimation. There was a slight increase in Fv/Fm during cold acclimation at 2°C in the early autumn period (data not shown).This indicates that the photochemical mechanism of photoacclimation, which was induced during pre-acclimation, further increased the tolerance to cold-induced photoinhibition during cold acclimation.

Temperature and daylength effect on freezing tolerance

The treatments under the late autumn period and highest acclimation temperature (12°C) had the lowest freezing tolerance for both species and populations. We have shown that a rise in pre-acclimation temperature (9, 12, 15°C) under controlled light conditions decreased both cold acclimation capacity and photoacclimation in the same populations (Dalmannsdottir et al. 2016). Malyshev et al. (2014) found temperature to be a stronger trigger of cold acclimation than photoperiod in an experiment with the grass species *Arrhenatherum elatius*. In the current study, northern-adapted populations had higher freezing tolerances than southern-adapted except at the shortest photoperiod and the highest temperature where there were no differences in freezing tolerance. This indicates that today's northern-adapted breeding material may lose its advantages over southern-adapted in the future climate.

Freezing tolerance was reduced in plants at the late autumn period compared to the early and the intermediate period. Treatment at later autumn light conditions includes reduction in the total irradiation energy, and higher light intensity or irradiance is known to increase cold acclimation in perennial ryegrass (Pollock et al. 1988, Harrison et al. 1997). Light intensity is even more important for cold acclimation than photoperiod (Lawrence et al. 1973). In a pilot study in autumn 2011, the freezing tolerance of timothy cv. 'Grindstad' at 6°C, grown under natural light at the same periods as used in the current study, was tested. Plants in the pilot study expressed gradually lower freezing tolerance when acclimated at later autumn light conditions (data not shown), significantly different

between all three autumn periods. In the current study, there were no differences between the early and the intermediate autumn period regarding freezing tolerance. The plant populations were not exactly the same as in the pilot study, and more importantly, yearly fluctuations in the amounts of clouds cause differences in irradiance and affect the cold acclimation process. Since we tested effects of natural light conditions during autumn, the effects of irradiance and day length are confounded and cannot be separated in this study. It is likely that the reduction in irradiance is even more important than short days in relation to reduced freezing tolerance of the populations.

We did not find any significant difference in freezing tolerance between 6 and 9/3°C. Studies of Sjøseth (1971) support these findings, but Eagles and Williams (1992) found that high day and low night temperatures (10/2°C) gave a positive effect on freezing tolerance of perennial ryegrass compared to a constant temperature (10°C). The effect of diurnal temperature differences on cold acclimation seems to be a complicated interaction between day length, light quality and intensity.

Timothy is known to be considerably more winter-hardy than perennial ryegrass (Sjøseth 1971, Jørgensen et al. 2010), but this was not the case in the present study, possibly because plants did not reach maximum seasonal hardening after only five weeks of acclimation treatments. On the other hand, the freezing tolerance capacity *per se* does not seem to be the limiting factor for poor survival of perennial ryegrass at in northern high latitude areas. Other factors involved in seasonal adaptation like inadequate growth cessation (Østrem et al. 2014), low non-structural carbohydrate accumulation during winter (Østrem et al. 2011), low resistance to ice encasement (Höglind et al. 2010) and susceptibility to fungal diseases (Hofgaard et al. 2003) may contribute more to the poor winter survival than freezing tolerance.

Photoacclimation processes responded more strongly to photoperiod than to temperature whereas freezing tolerance responded more to temperature than photoperiod. Both photoacclimation (photochemical quenching) and freezing tolerance was reduced with decreasing autumn light conditions and increasing temperature. In studies by Rapacz et al. (2004), winter survival of *Festulolium* genotypes correlated with increased energy dissipation and lower photosynthetic activity of PSII before winter.

Our results indicate that the projected climate change in the north may reduce freezing tolerance in grasses because plants will be pre-acclimated at higher temperatures and shorter day length. Current adapted breeding populations may have unacceptable freezing tolerance in future climate. The present species and cultivars may therefore have to be replaced by species and cultivars, which are able to acclimate adequately under new day length × temperature combinations, combinations which are unique in the global context. Future breeding programs for northern high-latitude areas will need

adapted germplasm and introgression of southern-adapted material in order to produce high yielding and persistent grass cultivars adapted to the future climates.

Author contributions

Dalmannsdottir, Jørgensen, Rapacz and Rognli designed, guided or participated in performing the experiment. Dalmannsdottir wrote the first draft and corrected the manuscript. Østrem and Larsen provided the plant material. Rødven did most statistical analysis and wrote the chapter on statistical analysis. All co-authors discussed results, reviewed and corrected the manuscript.

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Fig. 1. Experimental setup including global irradiation (Wm^{-2}) (solid line) during experimental period in year 2012. A sum of radiation for each light period during autumn (early, intermediate, and late) is presented. Day length (hours) in Tromsø (dotted line).

Fig. 2. Timeline for measurements. Time points for measurement of; (a) above ground biomass, (b) chlorophyll fluorescence and (c) freezing tolerance. Leaf elongation was measured every week during pre- and cold acclimation treatments. Plants were propagated from seeds at 20°C under 24 h photoperiod and 150 µmol m^{-2} s^{-1} artificial light. Pre-acclimation treatments were 6, 9/3°C (12 h day/12 h night) and 12°C under natural light. Cold acclimation (C) at 2°C was carried out under natural light conditions.

Fig. 3. Estimated values of leaf elongation (mm) for perennial ryegrass (PRG) and timothy measured every week during 4 weeks of treatment with pre-acclimation temperatures 6, 9/3°C (12 h day/12 h night) and 12°C, and 1 week of treatment with cold acclimation at 2°C for early, intermediate and late autumn period. Real value raw data included, 12°C (cross), 9/3°C (triangle), 6°C (circle).

Fig. 4. Changes in fluorescence parameters in southern and northern-adapted populations of perennial ryegrass and timothy measured after the three different pre-acclimation temperature treatments. Estimated mean values with 95% confidence intervals for full parametric modell are presented. E=Early period, I=intermediate period, L=Late period

Fig. 5. Survival of plant populations as a function of freezing temperature (°C) in a freezing test at the end of the experiment. The line at 50% survival indicates the LT_{50} value for the population. Predicted values are presented for each temperature treatment (6, 9/3, 12°C) and each light period during autumn (early, intermediate, late). Empirical proportions of survival at different freezing temperature are shown with dots, 12°C (cross), 9/3°C (triangle), 6°C (circle).