Theor. Exp. Plant Physiol. (2017) 29:25–36
DOI 10.1007/s40626-016-0079-1

The interactive effects of temperature and moisture on nitrogen fixation in two temperate-arctic mosses

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Received: 27 July 2016 / Accepted: 11 October 2016 / Published online: 28 February 2017

Abstract Nitrogen (N) fixation in moss-cyanobacteria associations is one of the main sources of 'new' N in pristine ecosystems like subarctic and arctic tundra. This fundamental ecosystem process is driven by temperature as well as by moisture. Yet, the effects of temperature and moisture stress on N_2 fixation in mosses under controlled conditions have rarely been investigated separately, rendering the interactive effects of the two climatic factors on N_2 fixation unknown. Here, we tested the interactive effects of temperature and moisture on N_2 fixation in the two most dominant moss species in a temperate heath, subarctic tundra and arctic tundra: *Pleurozium schreberi* and *Tomentypnum nitens*. Mosses with different moisture levels (25, 50, 100%) were kept at different temperatures (10, 20, 30 °C) and N_2 fixation was measured at different times after exposure to these conditions. *T. nitens* had the highest nitrogenase activity and this increased with moisture content, while effects were moderate for *P. schreberi*. Nitrogenase activity increased with temperature in all mosses, and the temperature optimum (T_{opt}) was between 20 °C and 30 °C for all mosses. Quick acclimatization towards higher temperatures occurred. Our results suggest that the contemporary and not the historical climate govern the response of moss-associated N_2 fixation to changes in the abiotic environment. Thus, climate change will have substantial impacts on N_2 fixation in dominant mosses in temperate, subarctic and arctic habitats.

Keywords Acetylene reduction · Arctic · Climate change · Cyanobacteria · Drought · Heathland

1 Introduction

Nitrogen fixation by moss-cyanobacteria associations is a main source of 'new' N for ecosystems like boreal forests, and subarctic and arctic tundra (DeLuca et al. 2002; Stewart et al. 2011; Zielke et al. 2005). Several moss species have been found to be colonized by N_2 fixing cyanobacteria, *Nostoc*, *Cylindrospermum* and *Stigonema* being the most common cyanobacterial genera (Ininbergs et al. 2011). Moss-associated N_2 fixation can contribute 1–3 kg N ha^{-1} $year^{-1}$ to total ecosystem N input in pristine, unpolluted areas (Rousk et al. 2015; Rousk and Michelsen 2016), exceeding atmospheric N deposition in these ecosystems (e.g.

Electronic supplementary material The online version of this article (doi:10.1007/s40626-016-0079-1) contains supplementary material, which is available to authorized users.

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Gundale et al. 2011). Yet, fixed N_2 by moss-associated cyanobacteria is not readily available to the rest of the ecosystem. Release of N from the moss carpet is slow; it can take several months before fixed N_2 is transferred to the soil (Rousk et al. 2016).

Moss-associated N_2 fixation is strongly affected by abiotic factors. Nitrogen deposition (Gundale et al. 2011; Ackermann et al. 2012; Rousk et al. 2013a), temperature (Smith 1984; Gentili et al. 2005; Rousk and Michelsen 2016) and moss-moisture content (Jackson et al. 2011; Rousk et al. 2014, 2015) are the most important factors controlling N_2 fixation activity in mosses. The abiotic factors likely act upon N_2 fixation in a hierarchical and interacting way, and moisture has been put forward as the most important driver of N_2 fixation (Belnap 2001). However, given that mosses dry out quickly at high temperatures (>25 °C for several hours; e.g. Rousk et al. 2014), temperature and moisture are likely strongly interacting to affect moss and colonizing cyanobacteria.

Although the effects of these abiotic factors on moss-associated N_2 fixation have been investigated previously (e.g. Jackson et al. 2011), hardly any experiments have been conducted under controlled conditions that combine the major drivers of N_2 fixation—moisture and temperature—in one experiment, despite their strong interconnection. For instance, Gundale et al. (2012a) found that an increase in air temperature of +2 °C combined with less frequent precipitation reduced N_2 fixation in feather moss *Pleurozium schreberi*. However, the effects on N_2 fixation were dependent on the sampling date, and the precipitation quantity had no effect. Thus, we still lack a conclusive assessment of the combined effects of temperature and moisture on N_2 fixation, especially at higher temperatures that lead to desiccation of the moss-host.

While free-living cyanobacteria can be active and fix N_2 at temperatures as low as −1 °C, the minimum temperature for activity can even be lower (−5 °C) when living in associations with lichens (Englund and Meyerson 1974). Soil cyanobacteria and cyanolichens can fix N_2 within a large temperature range (−5 and 30 °C, Belnap 2001), and the optimum temperature for N_2 fixation in free-living cyanobacteria from the Arctic, from temperate grasslands soils and from subalpine soils is between 20 °C and 30 °C (Jones 1977, Stewart et al. 1977; Coxson and Kershaw 1983). The free-living cyanobacteria *Stigonema,* collected in Brazil, had a maximum nitrogenase activity at 15–25 °C and declined at 35 °C, with no nitrogenase activity at 0 °C (Isichei 1980). These findings suggest that the response of N_2 fixation to increased temperature is not shaped by the historical climate, but rather by the contemporary conditions. A N_2 fixer community that is performing best at contemporary climatic conditions that differ from the historic conditions is likely a community that is not driven by the historic climate and visa versa.

The optimum growth temperature for most mosses is around 20 °C (Dilks and Proctor 1975), but the majority of mosses have a heat tolerance of 39–45 °C (Glime 2007) and they can survive high temperatures in a state of desiccation (in dormancy), in which their temperature tolerance is higher (Hearnshaw and Proctor 1982). Cyanobacteria are only active when sufficiently moist, and highest N_2 fixation rates in mosses are found at optimal moist conditions (Zielke et al. 2005; Jackson et al. 2011; Rousk et al. 2014, 2015). Similarly, N_2 fixation in free-living cyanobacteria increased with moisture content (Rousk et al. 2015). However, the moisture dependence of N_2 fixation can differ between habitats, likely as a result of adaptations, and a completely hydrated status may lead to glucose efflux, depleting the energy reserves necessary for N_2 fixation (Kershaw 1985). Mosses hosting cyanobacteria are found in a range of ecosystems like subarctic and arctic tundra as well as in temperate habitats. Given the varying climatic conditions these associations are found in, e.g. low precipitation and low temperatures in arctic habitats, with large temperature fluctuations, and a less fluctuating climate in temperate habitats, the long-term exposure to these different climatic regimes could have led to different acclimatisation of N_2 fixation to the historic climate (see e.g. Strickland et al. 2015).

Here, we report results from a temperature by moisture experiment in which we exposed two different feather moss species (*P. schreberi*, *Tomentypnum nitens*) from three ecosystems (temperate heath, subarctic birch forest, arctic tundra) to different temperatures (10 °C, 20 °C and 30 °C) and moisture levels (25%, 50% and 100% moss water content) to assess their interactive effects on N_2 fixation. The differences in the historic climate between the sites—cold and dry in the arctic site vs. milder and more moist in the temperate site, and intermediate conditions at the subarctic site—will likely harbour

differently adapted cyanobacterial communities, resulting in different responses of N_2 fixation towards changes in temperature and moisture. We selected the moss species that covered the largest fraction of the ground in each ecosystem. Furthermore, we tested if moss-associated N_2 fixation can acclimatize to a new temperature and how this acclimatization is altered when exposed to yet another temperature. We hypothesized that (1) N_2 fixation in all moss species increases with increasing moss moisture content, and N_2 fixation in mosses collected in the Arctic will be higher at lower moisture levels than in moss from the temperate heath and similar to the moss collected in the Subarctic, (2) N_2 fixation increases with increasing temperature, and N_2 fixation in moss from the temperate heath will peak at higher temperatures than in mosses from the arctic regions, and (3) N_2 fixation can acclimatize to new temperatures, and the acclimatization in mosses from the arctic habitats is quicker than in moss from the temperate heath.

2 Material and methods

2.1 Sampling sites

The feather moss *P. schreberi* was collected in a subarctic birch forest (also referred to as subarctic tundra) close to the Abisko Scientific Research Station, Northern Sweden. The mean annual air temperature in Abisko is 0.2 °C (30-year mean 1986–2015, ANS 2016). Mean annual precipitation is 337 mm (30-year mean 1986–2015, ANS 2016). The same moss species (*P. schreberi*) was collected 50 km NW of Copenhagen, Denmark, from a temperate heathland. The mean precipitation for the area (1961–1990) is 613 mm distributed on 113 days throughout the year. The mean annual air temperature for the same period is 8 °C [Danish Meteorological Institute 2013 (www.dmi.dk)]. Due to the low cover of *P. schreberi* at our arctic site, we instead selected the most abundant feather moss found in such settings, *T. nitens*. Samples of *T. nitens* were collected from an arctic tundra site on Disko Island, Greenland. This area has a typical low-arctic climate with a mean annual air temperature of −3.0 °C (1992–2012). The mean annual precipitation is 436 mm. Mosses were sampled in February, May and September for the temperate, the subarctic and the arctic sites, in an attempt to sample under similar temperatures despite contrasting environmental conditions. All mosses were kept in climate chambers at 10 °C in light (18 h) and at 2 °C in dark (6 h)—reflecting field conditions at the sampling dates, and also ensuring similar conditions for all mosses one month prior to the start of the experiments.

2.2 Sample preparation

All mosses were gently soaked in ddH_2O for 5 min to ensure water saturation (100%) and 3.0 ± 0.1 g of wet moss were put into 50 mL centrifuge tubes. Although we did not check cyanobacterial presence on the mosses before and after the soaking, we assume that the gentle soaking did not affect the cyanobacterial presence. Only alive, green parts of the mosses were used. The mosses from the temperate heath were rinsed thoroughly with ddH_2O before water saturation so that any deposited N, which could inhibit N_2 fixation (e.g. Rousk et al. 2013a), would be washed away. To reach the moisture levels below 100% moss water content (25% and 50%), mosses were slowly dried with a cold airflow. Moss samples from each site with three moisture levels (25%, 50% and 100% moss water content) were placed in climate chambers with three different temperatures (10 °C, 20 °C and 30 °C). Five replicate samples were used for each treatment from each site, totalling to 45 samples per temperature (5 samples per moisture level per site). Water loss was checked three times per week and adjusted accordingly.

2.3 Temperature by moisture experiment

The temperature by moisture experiment was carried out 1.5 and 2.5 weeks after the mosses had reached the moisture levels we aimed for. The experiment consisted of 45 samples in each temperature (10 °C, 20 °C and 30 °C). Five replicates per temperature and per sampling site were kept at three different moisture levels (25%, 50% and 100% moss water content). We used different incubation times for moss samples exposed to the different temperatures, which was a necessity given the expected dependence of nitrogenase activity on temperatures. Assuming a Q_{10} of 2.5 for N_2 fixation in mosses (see Smith 1984), we incubated the moss samples in the 10 °C, 20 °C and 30 °C for 20 h, 7.5 h and 3 h, respectively.

2.4 The interactive effects of incubation-temperature and -time on acetylene reduction

To test if acetylene reduction (see below) is affected by the incubation time with acetylene, depending on the incubation temperature, we incubated mosses from the 20 °C and 30 °C climate chamber for 20 h and another set of samples from the 30 °C climate chamber for 7.5 h. For these incubations, we used two moisture levels (50% and 100% moss water content) with five replicates from each site, temperature and moisture level. Acetylene reduction in samples from both the temperature by moisture and the incubation experiment was measured after 1.5 and 2.5 weeks kept at the aimed moisture levels.

2.5 Exposure to a higher temperature

To test if N_2 fixation in moss-cyanobacteria associations can acclimatize to a higher temperature within a short time frame, we moved the samples from 10 °C and 20 °C to 30 °C ($n = 5$ per moisture level, per site). For the acetylene reduction assay (ARA), samples were incubated for 3 h and ARA was performed 1 day, 1 week and 2 weeks after the move to the higher temperature.

2.6 Exposure to a lower temperature

Similarly, to test if N_2 fixation in moss-cyanobacteria associations can acclimatize to a lower temperature within a short time frame, the samples from the incubation temperature and -time experiment were moved to a lower temperature. One set of samples from the 30 °C treatment were moved to 20 °C and another set of samples was moved to 10 °C. The samples from the incubation temperature and –time experiment from the 20 °C treatment were moved to 10 °C. The incubation times for the samples in the 20 °C climate chamber was 7.5 and 20 h for the samples in 10 °C. Acetylene reduction was measured 1 day, 1 week and 2 weeks after the change to the lower temperature.

2.7 Acetylene reduction assay (ARA)

To estimate N_2 fixation, we used the acetylene reduction assay. This assay is a measure of the nitrogenase enzyme activity that catalyzes N_2 fixation. In all experiments (see above), 10% of the headspace of the 50 mL tubes was exchanged with acetylene gas. After the incubations, 6 mL air was taken from each sample and injected into 6 mL pre-vacated exetainer (Labco, Ceredigion, UK) and were analysed for ethylene production on a gas chromatograph (SRI 310C, FID, SIR Instruments, California).

2.8 Cyanobacterial counts on moss leaves

Cyanobacterial cells were counted in the samples from the temperature by moisture experiment, using an Olympus fluorescence microscope, with 200× to 400× magnification. Moss leaves from 3 replicate samples (1 sample = one 50 mL tube) from each treatment were counted. From each sample, we randomly picked five shoots, and from each shoot we took 3 fronds ("branches"). The leaves were scraped off the fronds with a needle, and transferred to a microscope-slide. From each frond, we counted 5 randomly selected leaves, totalling 75 leaves per sample.

2.9 Moss-pH

The pH of the mosses was assessed using three samples from each site (3 g wet weight). Moss samples were cut into small pieces and 15 mL ddH_2O was added. The samples were shaken for 1 h on a table shaker and centrifuged for 10 min with 4300 rpm. The pH was measured with a pH meter.

2.10 Moss N content

At the end of the experiment, moss samples were dried (70 °C for 24 h), ground and 3–4 mg were packed into tin capsules to determine the total N concentration on an Isoprime isotope ratio mass spectrometer (Isoprime Ltd., Cheadle Hulme, UK) coupled to a CN elemental analyzer (Eurovector, Milan, Italy).

2.11 Statistical analyses

To test the effects of temperature on nitrogenase activity, we performed analyses of Covariance (ANCOVA) for each site and time point separately, with moisture as the covariate. This enabled us to compare the slopes (acetylene reduction in relation to

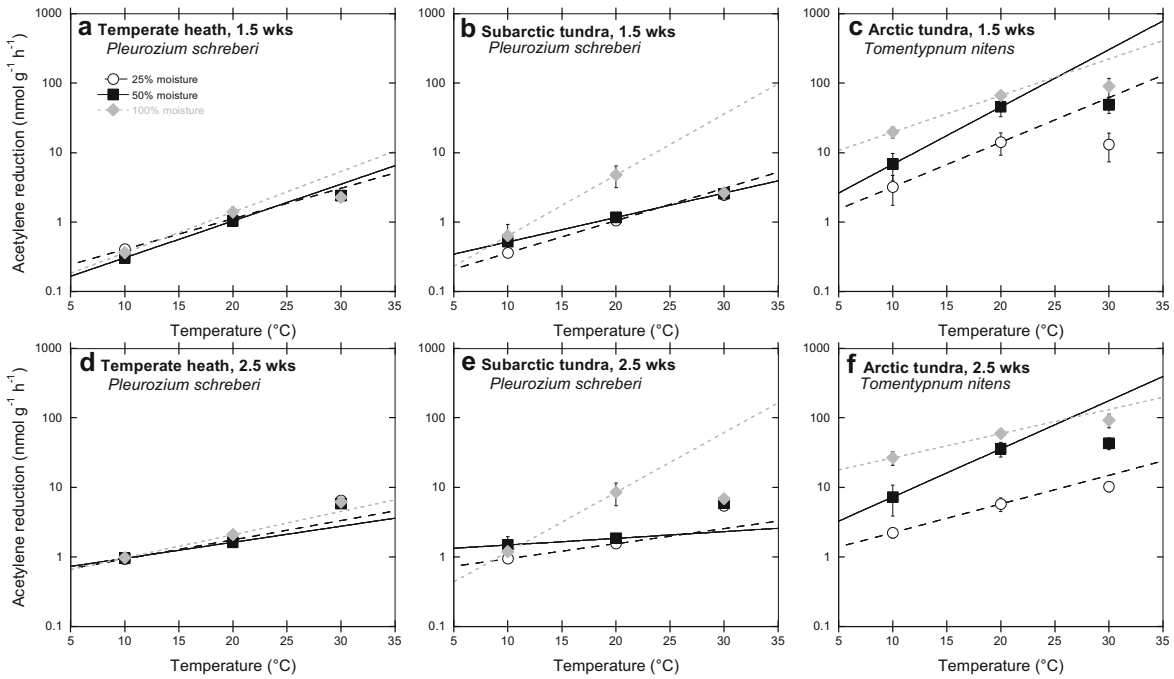

Fig. 1 Acetylene reduction (nmol g dw^{-1} h^{-1}) in *P. schreberi* from a temperate heath and subarctic tundra, and *T. nitens* from arctic tundra exposed for 1.5 (**a**–**c**) and 2.5 (**d**–**f**) weeks to three different moisture levels (open circles, black squares, grey diamonds for the 25%, 50% and 100% moss water content, respectively) within three temperature levels (10 °C, 20 °C, 30 °C). The lines were fitted through the mean acetylene reduction in the 10 °C and 20 °C treatments and thereafter extended to the axes. Data for the 30 °C treatment was plotted separately. Shown are mean ± SE (n = 5). Please note the logarithmic y-axis

exposure temperature) between the different moisture levels. Acetylene reduction data were log-transformed to fulfil the assumptions of ANCOVA. ANCOVA's were performed for the data from the temperature by moisture experiment as well as for the change in the exposure experiments. Changes in ARA with incubation times in the incubation temperature and -time experiment as well as changes in Q_{10} values with time were assessed with linear regression analyses. Differences in moss-pH and moss N content between sites were assessed with ANOVA. All analyses were performed in R. 3.0.3.

3 Results

3.1 Temperature by moisture experiment

Acetylene reduction increased from 10 °C to 20 °C in all mosses 1.5 and 2.5 weeks after exposure to the moisture and temperature levels (Fig. 1a, b) ($F > 27$; $p < 0.0001$ for all mosses). The highest AR activity was found in *T. nitens* ($F = 34.17$; $p < 0.0001$), irrespective of temperature and moisture level. While AR activity in *P. schreberi* from both sites (subarctic and temperate) at 30 °C overlapped with the fitted line for the 10 °C and 20 °C data after 1.5 week of exposure to the moisture and temperature levels, nitrogenase activity acclimatized after 2.5 weeks and the temperate optimum seems to be above 30 °C (Fig. 1d, e). Nitrogenase activity in *T. nitens* however, did not seem to change over time and 30 °C was above the temperature optimum throughout (Fig. 1c, f). Moisture had a positive effect on acetylene reduction in *P. schreberi* form the subarctic site ($F_{1.5\ wks} = 6.06$; $p_{1.5\ wks} = 0.02$; $F_{2.5\ wks} = 8.3$; $p_{2.5\ wks} = 0.006$) and *T. nitens* ($F_{1.5\ wks} = 37.3$; $p_{1.5\ wks} < 0.0001$; $F_{2.5\ wks} = 90.1$; $p_{2.5\ wks} < 0.0001$), but there was no interaction with temperature. Nitrogenase activity in *P. schreberi* from the Subarctic at 20 °C and 100% moisture content was variable and high, also indicated by the variable numbers of cyanobacteria on the moss leaves (Online Resource 1). Acetylene reduction increased with time in *P. schreberi* from the subarctic ($R^2 = 0.14$; $p = 0.0002$) and temperate site ($R^2 = 0.20$; $p < 0.0001$).

Table 1 Q_{10} for acetylene reduction in the mosses *P. schreberi* from a temperate heath and subarctic tundra, and *T. nitens* from arctic tundra exposed to different moisture levels (25%, 50% and 100% moss water content) and temperatures (10 °C, 20 °C, 30 °C)

Moisture (%)	Site	10 → 20					20 → 30				
		T1 Exp. 1	T2 Exp. 1	T1 Exp. 2	T2 Exp. 2	T3 Exp. 2	T1 Exp. 1	T2 Exp. 1	T1 Exp. 2	T2 Exp. 2	T3 Exp. 2
25	Temperate heath	2.75	1.89	0.83	0.87	1.01	2.17	3.75	1.43	1.07	1.02
	Subarctic tundra	2.92	1.65	0.68	0.85	0.97	2.34	3.53	1.43	1.11	1.05
	Arctic tundra	4.40	2.58	0.98	0.84	1.53	0.94	1.80	1.65	3.42	1.32
50	Temperate heath	3.39	1.70	0.86	0.84	0.94	2.33	3.61	1.29	1.12	1.06
	Subarctic tundra	2.25	1.25	0.78	0.93	0.93	2.20	3.24	1.29	1.03	1.07
	Arctic tundra	6.68	4.93	1.32	0.85	0.84	1.09	1.22	1.24	1.47	1.42
100	Temperate heath	3.85	2.16	0.85	0.88	0.83	1.65	2.98	0.33	0.99	1.12
	Subarctic tundra	7.51	7.21	3.17	2.30	2.28	0.56	0.82	0.33	0.36	0.43
	Arctic tundra	3.36	2.23	0.83	0.71	1.12	1.38	1.59	1.03	1.07	1.13

Given are the Q_{10} values for the increase in the acetylene reduction rate for a temperature change from 10 °C to 20 °C and from 20 °C to 30 °C and for all measured time points in the two experiments. Experiment 1: moss samples were exposed to 10 °C, 20 °C and 30 °C; experiment 2: moss samples were exposed to 30 °C from previously 10 °C and 20 °C and the moss samples from 30 °C were kept at 30 °C. T1 Experiment 1 = 1.5, T2 Experiment 1 = 2.5 weeks exposed to the climatic conditions. T1, T2, T3 Experiment 2 = 1 day, 1 week and 2 weeks after exposure to the abiotic climatic conditions, respectively

The Q_{10} values ranged from 0.6 to 7.5 in *P. schreberi* and from 0.9 to 6.7 in *T. nitens* (Table 1). Nitrogen fixation in these mosses was more sensitive to changes (i.e., higher Q_{10}) in temperature from 10 °C to 20 °C than in a change from 20 °C to 30 °C (Table 1). The Q_{10} values decreased with time and increased with moisture content ($p < 0.006$ for all mosses), except for the change from 20 °C to 30 °C in *P. schreberi* from the temperate site and in *T. nitens*.

3.2 Incubation-temperature and -time experiment

The different incubation times depending on exposure temperature had a large effect on nitrogenase activity (Fig. 2), with lower activity in the longer incubations in all moss species ($R^2 = 0.79$; $p < 0.0001$).

3.3 Exposure to a higher temperature

The original temperature had an effect on acetylene reduction in all mosses shortly after exposure to the higher temperature (30 °C). Mosses that had been kept at 30 °C throughout had higher nitrogenase activity than mosses previously exposed to 10 °C and 20 °C ($F = 5.07$; $p = 0.02$) (Fig. 3). *T. nitens* had the highest nitrogenase activity, and *P. schreberi* from the Subarctic had higher activity than the same species from the temperate heath ($F = 380.2$; $p < 0.0001$). The highest moisture levels in the subarctic moss resulted in higher nitrogenase activity ($F = 6.1$; $p = 0.02$) and acetylene reduction increased with increasing moisture level in *T. nitens* ($F = 37.3$; $p < 0.0001$). However, no effect of moisture on nitrogenase activity was observed in *P. schreberi* from the temperate heath. Acetylene reduction was lowest shortly (1 day) after the move to 30 °C ($F = 8.81$; $p = 0.0002$) (Fig. 3). The data points from *P. schreberi* kept at 30 °C are above the line extrapolated from the 10 °C and 20 °C data after 1 day and 1 week, indicating that 30 °C is above the temperature optimum for the samples that were previously kept at the lower temperatures (Fig. 3a, b, d, e). However, after 2 weeks exposure to 30 °C, the data points fall on the fitted line, indicating that nitrogenase activity in the samples from 10 °C and 20 °C acclimatized to the new, higher temperature (Fig. 3g, h). The temperature optimum for acetylene reduction in *T. nitens* seemed to change with time (Fig. 3c, f, i), and after 2 weeks, the data points for the 30 °C samples fitted the extrapolated line from the 10 °C and 20 °C samples at 25% water content. This indicates that the mosses from the colder temperatures acclimatized to 30 °C.

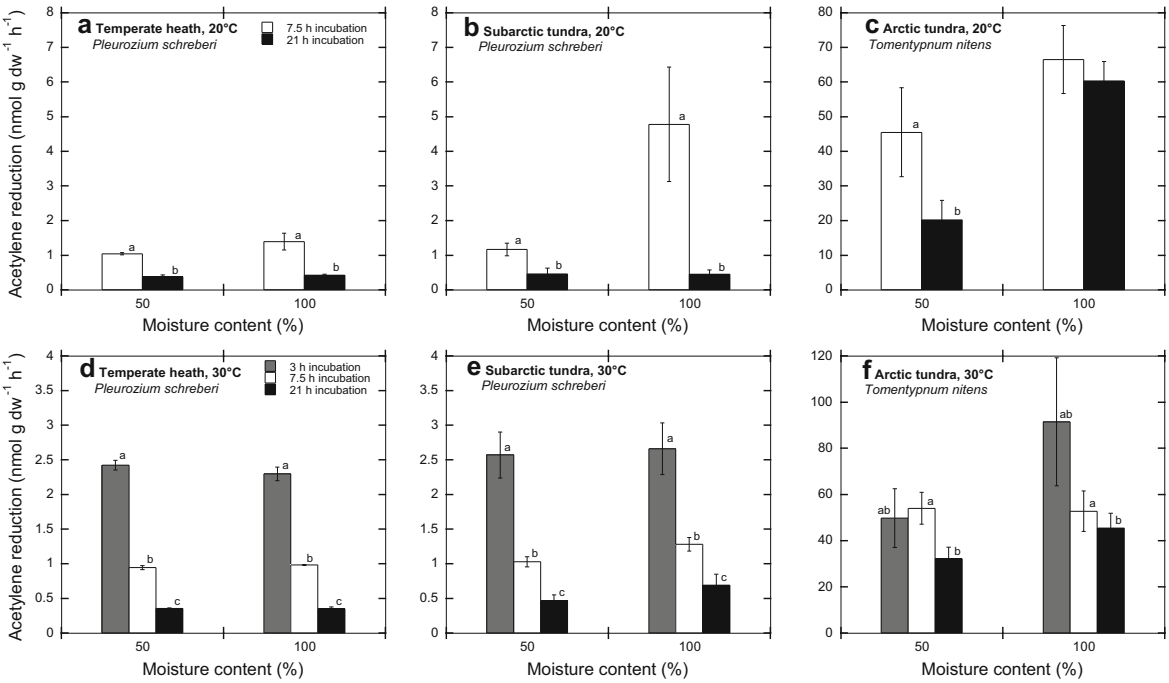

Fig. 2 Acetylene reduction (nmol g dw^{-1} h^{-1}) in *P. schreberi* from a temperate heath and subarctic tundra, and *T. nitens* from arctic tundra incubated for different times with acetylene. Shown are mean values (n = 5) ± SE of acetylene reduction in mosses exposed for 1.5 weeks to two different moisture levels (50%, 100% moss water content) within two temperature levels (20 °C, 30 °C). The samples were incubated for 7.5 (*white bars*) and 21 h (*black bars*) at 20 °C and for 3 (*grey bars*), 7.5 and 21 h at 30 °C. Please note the differences in the y-axes. Different lower case letters indicate significant differences between the incubation times within a moisture level (Tukey's test)

3.4 Exposure to a lower temperature

Acetylene reduction in *P. schreberi* from the temperate heath changed over time, ($F = 20.71$; $p < 0.0001$), and was highest in the 20 °C samples ($F = 45.4$; $p < 0.0001$) and the effects of temperature was dependent on the time of exposure to the new temperatures ($F = 49.89$; $p < 0.0001$), but not on the moisture level of the moss (Online Resource 2a). Similarly, acetylene reduction in *P. schreberi* from the Subarctic changed over time ($F = 4.65$; $p = 0.01$), and was highest in the samples that have been moved from 30 °C to 20 °C ($F = 45.6$; $p < 0.0001$) (Online Resource 2b). The effects of temperature were dependent on the time ($F = 6.49$; $p = 0.003$) and on the moisture level of the mosses ($F = 11.86$; $p = 0.0009$). Nitrogenase activity in *T. nitens* was lowest shortly after the samples were moved to the new temperatures ($F = 8.23$; $p = 0.0006$). Moisture had a positive effect on acetylene reduction ($F = 38.71$; $p < 0.0001$), and was highest in the mosses that were moved from 30 °C to 20 °C ($F = 362.8$; $p < 0.0001$). Further, moisture and temperature interacted to affect nitrogenase activity ($F = 4.57$; $p = 0.04$) (Online Resource 2c).

3.5 Moss-pH

The pH was highest in *T. nitens* (5.59 ± 0.07), and lower in *P. schreberi* from subarctic tundra (4.32 ± 0.009) than from the temperate heath (4.76 ± 0.09; $F = 94.73$; $p < 0.0001$) (Online Resource 3).

3.6 Moss N content

Pleurozium schreberi from the temperate heath had the highest N tissue concentrations (averaged across all treatments: 0.92 ± 0.04% N) at the end of the experiment, and *P. schreberi* from the Subarctic (0.65 ± 0.03% N) had higher tissue N content than *T. nitens* (0.54 ± 0.01% N; $F = 86.6$, $p < 0.00001$).

Total N content of mosses kept at 30 °C was higher than of mosses kept at 20 °C ($F = 3.44$, $p = 0.035$), as well as mosses with 25% moisture compared to mosses with 100% moisture ($F = 6.169$, $p = 0.014$) (Online Resource 4).

4 Discussion

4.1 Temperature by moisture experiment

We assessed the combined effects of temperature and moisture on N_2 fixation in two moss species, and how the response to the contemporary climatic conditions (i.e. temperature and moisture) is shaped by the climatic history. Our results suggest that the response of N_2 fixation in moss-cyanobacteria associations towards changes in temperature and moisture are shaped more strongly by the contemporary climatic conditions than by the historic conditions. Yet, exposure to extreme temperatures (30 °C for the arctic moss) seems to reveal the influence of the historic climate on N_2 fixation. Nitrogen fixation was influenced by moisture only in the moss from the Arctic, *T. nitens*, somewhat contradicting our first hypothesis. Although nitrogenase activity in *P. schreberi* from the Subarctic seemed to be affected by moisture levels, the effects were mostly driven by the moss samples at 20 °C, 100% moisture, which was very variable in cyanobacterial colonization, and with that, activity. Nonetheless, our study suggests that N_2 fixation is controlled in a hierarchical way (see e.g. Belnap 2001), and that the hierarchy differs between moss species and/or ecosystem. Nitrogen fixation in *P. schreberi* from both the temperate and subarctic site seem to be mostly driven by the temperature the colonized moss is exposed to, whereas activity in *T. nitens* seemed to be driven by both moisture and temperature. The differences in the response of N_2 fixation to the different abiotic factors between the samples could be due to differences between the moss species. *T. nitens* can hold water two times longer than *P. schreberi* (Elumeeva et al. 2011), which could explain the much higher cyanobacterial colonization and N_2 fixation rates in this moss. Even if we may not be able to ascertain if the differences were driven by the moss species or the ecosystem, our intent to deepen our understanding of the effects of climatic factors on moss-associated N_2 fixation at ecosystem level made it necessary to choose the most dominant and abundant species in the respective ecosystem. Nevertheless, our study gives us novel insights into the abiotic controls of moss-associated N_2 fixation.

The highest here investigated moisture level did not automatically translate into the highest nitrogenase activity, except for *T. nitens*, despite that moisture previously has been proposed as a fundamental driver of N_2 fixation (Belnap 2001; Rousk et al. 2014, Rousk and Michelsen 2016). However, moist moss is more sensitive to heat stress than dry moss (Glime 2007). When mosses are dry, their activity is low or they are in a dormant state. When mosses are optimally moist, all biological process rates are high, and more damage possible, which in turn can affect the colonizing cyanobacteria. Further, if the photosystem of mosses is damaged too severely, the energy transfer to the colonizing cyanobacteria might be compromised, limiting N_2 fixation. However, evidence of carbon (C) transfer from moss to cyanobacteria is still missing.

Temperature had a similar, positive effect on N_2 fixation in all mosses, corroborating our second hypothesis only partly. Our experiment suggests that 30 °C is not extreme enough to damage the moss, even when moist. Most bryophytes have an optimum for growth around 20 °C (Dilks and Proctor 1975), *Sphagnum* mosses being an exception with a growth optimum at 30 – 35 °C (Li and Glime 1990). However, the measured air temperature can be very different from bryophyte temperature and they can cool down via evaporation (Glime 2007), thus growth likely occurs at higher temperatures. Also, cell membrane permeability increases with increasing temperatures (Liu et al. 2003), which could lead to C and nutrient release (e.g. phosphorus; Carleton and Read 1991) at higher temperatures, benefitting the colonizing cyanobacteria.

Although the subarctic site in Northern Sweden receives low amounts of atmospheric N (0–2 kg N ha^{-1} $year^{-1}$) (Karlsson et al. 2009; Gundale et al. 2011), N_2 fixation was low and comparable to N_2 fixation in the same species from the temperate heath, which receives 11 kg N ha^{-1} $year^{-1}$ (Ellermann et al. 2011). While this might be below the N deposition threshold of nitrogenase activity in mosses (see e.g. Rousk et al. 2014), the higher N content of the moss from the temperate heath (Online Resource 4) likely reflects the exposure to higher N depositions rates (Harmens et al. 2014).

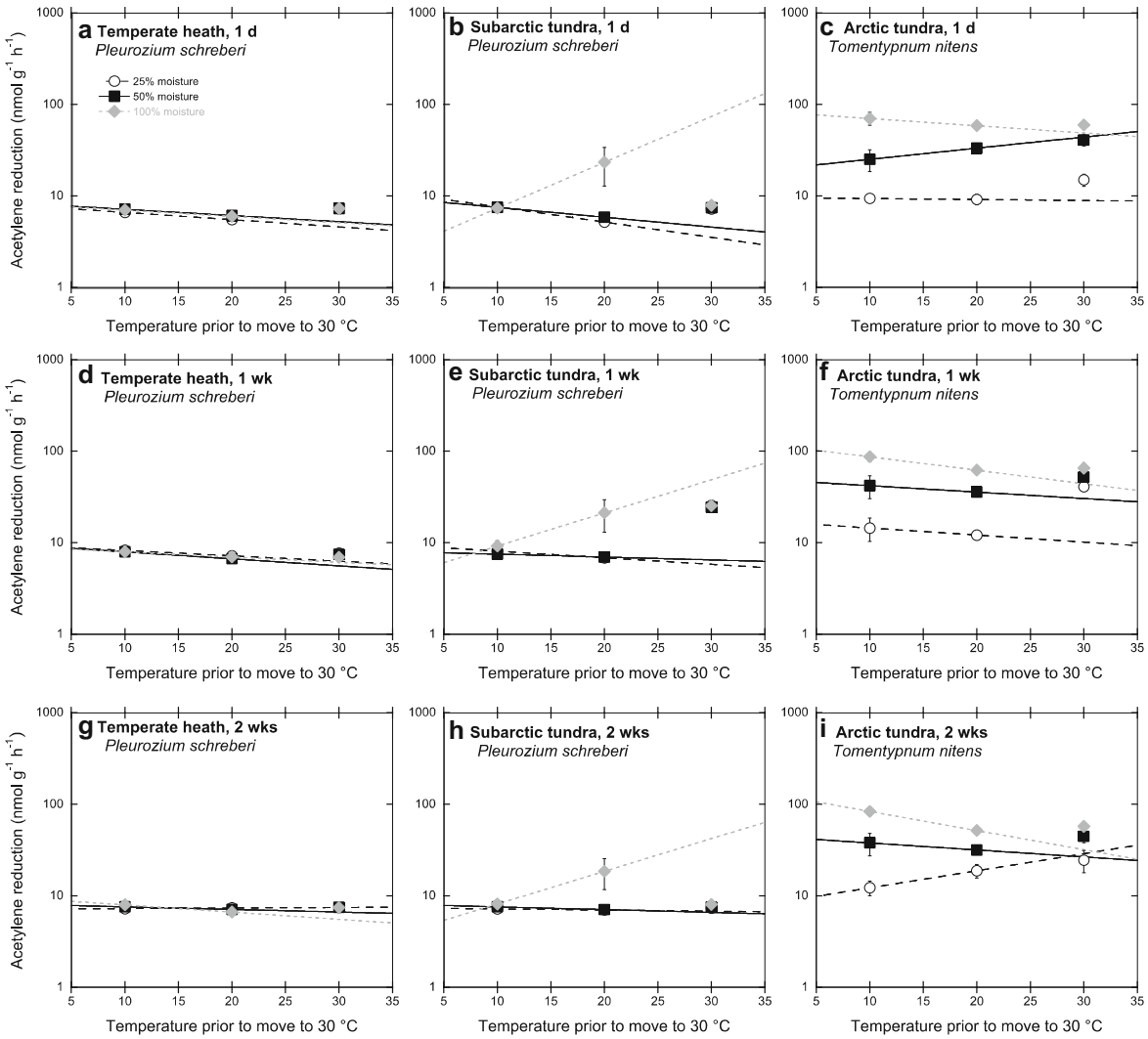

Fig. 3 Acetylene reduction (nmol g dw^{-1} h^{-1}) in *P. schreberi* from a temperate heath and subarctic tundra, and *T. nitens* from arctic tundra exposed for 1 day (**a**–**c**), 1 week (**d**–**f**) and 2 weeks (**g**–**i**) to three different moisture levels (open circles, black squares, grey diamonds for the 25%, 50% and 100% moss water content, respectively) within one temperature (30 °C). Samples from the 10 °C and 20 °C treatments were moved into 30 °C. The lines were fitted through the mean acetylene reduction in the 10 °C and 20 °C treatments and thereafter extended to the axes. Data for the 30 °C treatment was plotted separately. Shown are mean ± SE (n = 5). Please note the logarithmic y-axis

We observed several different species of cyanobacteria on *T. nitens*, which could have their activity optimum at different temperatures and moisture levels (see also Gentili et al. 2005), whereas *P. schreberi* from from the Subarctic as well as from the temperate heath harboured less diverse cyanobacterial communities. This hints to a wider niche breadth of N_2 fixation in *T. nitens* compared to *P. schreberi*. Future studies using molecular techniques could verify whether this is a general phenomenon.

4.2 Incubation-temperature and -time experiment

The experiment on the different incubation times demonstrates that the incubation time should be adjusted according to the incubation temperature. This is most likely the case for other enzyme assays as well, and not only for the acetylene reduction assay. With higher temperatures, biological process rates increase, and rates will be faster in the first hours and decrease over time. Thus, longer incubations (>20 h)

underestimate process rates at higher temperatures. Previous studies assessing temperature effects on nitrogenase activity failed to adjust the incubation times according to temperature, and may potentially have underestimated and thereby, misrepresented N_2 fixation rates. Furthermore, increased process rates lead to changes in the incubation conditions. For instance, rates of photosynthesis could increase which could lead to increased O_2 concentrations, potentially inhibiting N_2 fixation (Staal et al. 2001). Although more energy could be generated via higher photosynthesis rates, promoting N_2 fixation, the increased activity will likely level out over time in long incubations, and the average activity will then be lower than it actually was. We assumed a Q_{10} of 2.5 (Smith 1984) and calculated the incubation times for the different temperatures accordingly, which seemed to be adequate for N_2 fixation in mosses from the different ecosystems.

4.3 Exposure to higher and lower temperatures

The Q_{10} values indicate that N_2 fixation in all mosses is more sensitive to a temperature change between 10 °C and 20 °C than between 20 °C and 30 °C. Furthermore, the optimum temperature for N_2 fixation in both moss species seems to be between 20 °C and 30 °C and thus, falls within the kinetic optimum for nitrogenase (25 °C, Houlton et al. 2008). Nevertheless, lower temperature optima have been suggested for N_2 fixation in mosses (peak activity at 13 °C and 22 °C due to different cyanobacterial colonizers with different optima (Gentili et al. 2005; see also Gundale et al. 2012b). The Q_{10} values for N_2 fixation in *P. schreberi* from the Subarctic and *T. nitens* from the Arctic were variable and higher than for N_2 fixation in *P. schreberi* from the temperate heath. This indicates higher sensitivity to a change in temperature in the high latitude ecosystems. Here, mosses are exposed to large temperature fluctuations within a few hours, probably more so than the mosses from the temperate heath. This historical exposure to extreme temperature differences could make the cyanobacterial colonizers more sensitive to a change in temperature. Nevertheless, the temperature optimum for N_2 fixation in *P. schreberi* from both sites seems to increase within a few weeks of exposure to a different temperature, suggesting a capability for acclimatization. This corroborates our third hypothesis only partly since no acclimatization towards high temperatures could be observed in *T. nitens*. Acclimatization to high temperatures has been observed in bryophytes (e.g. in *Polytrichum commune*, Sveinbjörnsson and Oechel 1983) and they can increase their heat resistance if exposed to temperatures above their optimum temperature for growth (Antropova 1974). If increased temperatures do not necessarily inhibit moss growth, the cyanobacterial colonizers are also likely to be less affected by an extreme increase in temperatures. However, for *T. nitens*, 30 °C seems to be above the temperature optimum for N_2 fixation. This is not surprising given a mean annual temperature of −3 °C at the arctic site compared to 0.5 °C and 8 °C at the subarctic and temperate site, respectively.

After moving the mosses to a lower temperature, the highest nitrogenase activity was found in the change from 30 °C to 20 °C: activity was higher after the move from 30 °C into 20 °C than activity before the move. This indicates that a temperature closer to 20 °C is more beneficial for N_2 fixation in mosses than 30 °C. Although our extended curves suggest a higher temperature optimum of N_2 fixation, the maximum temperature is likely to be close to those values (Smith 1984; Gentili et al. 2005). The temperature decrease from 20 °C to 10 °C and from 30 °C to 10 °C resulted in similar nitrogenase activity. This suggests that the historical climatic conditions (temperature) are not as important as the contemporary conditions or that the mosses and colonizing cyanobacteria acclimatize quickly to the new conditions. Nitrogenase activity in mosses that had been moved from 10 °C and 20 °C to 30 °C seemed to acclimatize to the new temperature within a few days. However, activity was higher in those mosses before the move, indicating that the response to an increased temperature is adaptation whereas the response towards a lower temperature is acclimatization.

4.4 Moss-pH

The pH optimum for N_2 fixation in mosses is between 5.9 and 6.2 (Smith 1984) and even higher in free-living *Nostoc* (7 or above) (Granhall 1981). Thus, *P. schreberi*, with a pH below 5, might not provide the ideal conditions for the cyanobacterial colonizers. Yet, other results show that *P. schreberi* from boreal forests in Northern Sweden is highly colonized by cyanobacteria (Gundale et al. 2011, Rousk et al. 2013b, 2014),

suggesting a habitat effect rather than a moss species effect.

5 Conclusions

Nitrogen fixation has been assumed to be higher in warmer regions than in boreal forests, subarctic and arctic habitats due to temperature limitation in northern ecosystems. Yet, N_2 fixation does occur in the boreal biome, even at low temperatures and across a large range of temperatures as well as moisture levels, indicating a wide niche breadth of N_2 fixation associated with mosses. Given the here shown ability of N_2 fixation to acclimatize to an increase in temperature within a short time period in different moss species, ecosystem N input via moss-associated N_2 fixation will increase in a future climate, especially in mosses that are less sensitive to low moisture levels.

Acknowledgments Funding was provided by the Danish Council for Independent Research and FP7 Marie Curie Actions "COFUND" (Grant ID: DFF—1325-00025), as well as from the Danish Council for Independent Research "Research Project 1" (Grant ID: DFF—6108-00089), and the Danish National Research Foundation (Center for Permafrost, CENPERM DNRF100). We thank Gosha Sylvester and Maja Holm Wahlgren for assistance with laboratory analyses at the University of Copenhagen, and Abisko Scientific Research Station for logistics and access to climate data.

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