Four years of experimental warming do not modify the interaction between subalpine shrub species

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Abstract

Climate warming can lead to changes in alpine plant species interactions through modifications in environmental conditions, which may ultimately cause drastic changes in plant communities. We explored the effects of four years of experimental warming with open-top chambers (OTC) on *Vaccinium myrtillus* performance and its interaction with neighbouring shrubs at the Pyrenean treeline ecotone. We examined the effects of warming on height, above-ground (AG) and below-ground (BG) biomass and the C and N concentration and isotope composition of *V. myrtillus* growing in pure stands or in stands mixed with *Vaccinium uliginosum* or *Rhododendron ferrugineum*. We also analysed variations in soil N concentrations, rhizosphere C/N ratios and the functional diversity of the microbial community, and evaluated whether warming altered the biomass, C and N concentration and isotope composition of *V. uliginosum* in mixed plots. Our results showed that warming induced positive changes in the AG growth of *V. myrtillus* but not BG, while *V. uliginosum* did not respond to warming. *Vaccinium myrtillus* performance did not differ between stand types under increased temperatures, suggesting that warming did not induce shifts in the interaction between *V. myrtillus* and its neighbouring species. These findings contrast with previous studies in which species interactions changed when temperature was modified. Our results show that species interactions can be less responsive to warming in natural plant communities than in removal experiments, highlighting the need for studies involving the natural assembly of plant species and communities when exploring the effect of environmental changes on plant-plant interactions.

Keywords: dwarf shrub, plant interactions, Pyrenees, *Vaccinium myrtillus*, passive warming

Abbreviations: AG (above-ground), BG (below-ground), $\delta^{13}C$ (carbon isotope composition), $\delta^{15}N$ (nitrogen isotope composition)

Introduction

In recent decades, climate warming and land-use change (i.e. abandonment of extensive livestock grazing and tree logging) have led to shrub encroachment processes in the alpine treeline ecotone worldwide (Dullinger et al. 2003; Rundqvist et al. 2011; Ropars and Boudreau 2012). The forecasted global air temperature increase of 1.0–3.7 ºC by the end of the century could accelerate these processes; especially at high elevations and latitudes, where change is predicted to be larger (Nogués-Bravo et al 2007; Collins et al. 2013; Rangwala et al. 2013). This could have a dramatic impact on alpine and Arctic tundra ecosystems due to shifts in community composition and potential feedbacks to warming, such as decreasing albedo due to the higher radiation absorption by shrub canopies, reducing radiative cooling at night through the reflection of the thermal energy emitted by the soil, or through the inputs of more recalcitrant litter in the ecosystem (Hobbie 1996; Cornelissen et al. 2007; Myers-Smith et al. 2011; D'Odorico et al. 2013).

Many studies in Arctic and alpine ecosystems have shown the need to conduct species-specific studies to understand vegetation changes with warming, since coexisting species may differ in their responses to increasing temperatures (Kudo and Suzuki 2003; Klanderud 2008; Anadon-Rosell et al. 2014; Little et al. 2015; Yang et al. 2015). However, it is also important to consider plant–plant interactions, since they are crucial for plant community dynamics (Callaway and Walker 1997). The stress-gradient hypothesis (Bertness and Callaway 1994) postulates that competition is the major selective force in habitats with more benign environmental conditions, whereas facilitation dominates in more severe environments. Many studies in cold regions across the globe have shown that plant interactions shift from facilitation to competition as temperature increases, or in the opposite direction when temperature decreases (Shevtsova et al. 1997; Choler et al. 2001; Klanderud 2005; Pugnaire et al. 2015; Wheeler et al. 2015; Olsen et al. 2016). Nevertheless, most of these studies involved plant removal experiments, and studies focusing on the effects of temperature changes on plant interactions within natural communities are scarce (but see Dormann et al. 2004).

Shrubs are major components of alpine and Arctic tundra ecosystems. Amongst them, clonal dwarf shrub species are of great importance in terms of vegetation cover, structure and functionality. They present a complex network of subterranean rhizomes bearing fine roots, and producing individual above-ground (AG) ramets. Thus, the below-ground (BG) system of clonal shrubs is essential for their persistence and vegetative expansion, as well as an important source of soil carbon (C) (Cornelissen et al. 2014). Changes in the BG structure of dominant clonal shrubs could translate into major changes in the community and ecosystem functioning. Consequently, the study of BG responses to warming is an essential part of the complex responses to temperature increase in Arctic and alpine areas. However, the destructive nature of BG sampling and the difficulty to identify and separate roots from different species, together with the compromise of having studies running for the longest term possible, explain why warming experiments including both AG and BG plant measurements are infrequent (but see Hollister and Flaherty 2010 and Yang et al. 2015, amongst others).

Global warming may also induce shifts in the composition and function of the soil microbial community (Streit et al. 2014; Classen et al. 2015; DeAngelis et al. 2015), which can have strong impacts on ecosystem functioning (Schimel and Schaeffer 2012). For instance, rising temperatures can alter nitrogen (N) mineralization, with effects on N availability and, ultimately, plant growth (Bardgett and Wardle 2010). Several studies in cold ecosystems have found an increase in the soil N pool size with warming (Chapin et al. 1995; Hartley et al. 1999; Dijkstra et al. 2010; Dawes et al. 2011; Bai et al. 2013), which has been related to a stimulation of mineralization and decomposition processes. Since coexisting species show different N preferences and N-acquisition strategies (Körner et al. 2003; Pornon et al. 2007), shifts in N pools may affect interspecific interactions by altering relative niche and fitness differences between species (Chesson 2000; Tilman and Lehman 2001).

Vaccinium myrtillus L. forms shrub patches that colonize subalpine and alpine grasslands in the Pyrenees, where it grows close to the upper altitudinal limit of its distribution (Bolòs et al. 2005), subjected to low temperatures and short growing seasons. Warmer temperatures could favour its growth at the treeline ecotone, as has been reported in warming experiments in the Alps (Dawes et al. 2011; Anadon-Rosell et al. 2014) and in the Arctic tundra (Rinnan et al. 2009; Taulavuori et al. 2013). However, co-occurring species such as *Vaccinium uliginosum* or *Empetrum hermaphroditum* have not been found to respond to temperature increase (Richardson et al. 2002; Kudo and Suzuki 2003; Anadon-Rosell et al. 2014) and, consequently, interactions between these species might shift with warming. On the other hand, in line with the stress-gradient hypothesis, a modification of the environment through air temperature increase could induce changes in the interaction between this species and its neighbours towards increased competition. Despite the numerous studies focusing on *V. myrtillus* in tundra ecosystems, to our knowledge the potential effects of warming on the interaction with its neighbours have not been reported. Moreover, the previously mentioned experiments on *V. myrtillus* have mainly focused on its AG responses to warming, whereas BG effects have not been assessed.

At the treeline ecotone in the Central Pyrenees, *V. myrtillus* grows in pure patches (stands hereafter) or in mixed stands together with *Vaccinium uliginosum* L. subsp. *microphyllum* (Lange) Tolm. (hereafter *V. uliginosum*), or *Rhododendron ferrugineum* L. A previous study did not find evidence that the co-occurrence with these neighbouring shrubs had major effects on *V. myrtillus* structure and functioning (Anadon-Rosell et al. 2016). The objective of the present study was to investigate the AG and BG effects of four years of passive warming on *V. myrtillus*, and whether warming induced changes in interactions between *V. myrtillus* and its neighbouring species: a shrub of a very similar size (*V. uliginosum*) and a taller shrub (*R. ferrugineum*). For this purpose we assessed *V. myrtillus* phenology, AG and BG biomass, C and N concentration and isotopic signature ($\delta^{13}C$ and $\delta^{15}N$), soil inorganic N concentrations (NO_3^- and NH_4^+) and C and N availability in the rhizosphere in different types of *V. myrtillus* stands subjected to warming treatments. We also assessed the effects of warming on *V. uliginosum* AG biomass and C and N concentration and isotopic signature in mixed stands. Moreover, we evaluated the effects of warming on the functional diversity of the microbial community in *V. myrtillus* pure stands. We hypothesized that warming will (i) benefit *V. myrtillus* AG and BG performance (i.e. biomass and physiological traits), but that it will also (ii) modify interactions with co-occurring species, which will be manifested as different responses to warming of *V. myrtillus* when growing in pure stands than when growing in mixtures. We also hypothesized that (iii) the soil inorganic N pool will increase under warming with different magnitude across stand types.

Materials and methods

Study area

The study site was located at Eth Corrau des Machos (Val d'Aran), in the buffer zone of the Aigüestortes and Estany de Sant Maurici National Park (Central Pyrenees, Catalonia, UTM coordinates 31N 329, 472), on a N-facing 10-15º steep slope at 2250 m a.s.l. The vegetation consisted of *Festuca eskia* Ramond ex DC. and *Nardus stricta* L. grasslands mixed with patches of dwarf shrub heath dominated by *V. myrtillus*, *V. uliginosum* and *R. ferrugineum*. For the period 2001-2013, the mean annual precipitation and mean annual temperature (± SD) were 1146.4 (± 58.3) mm and 3.0 (± 0.2) °C, respectively. For the study period (2010-2013) the mean annual precipitation and mean annual temperature were 1223.1 (± 244.8) mm and 2.7 (± 0.9) °C, respectively. The mean monthly precipitation and the mean temperature for the main months of the growing season (June–August) were 99.1 (± 28.5) mm and 10.2 (± 1.0) ºC (obtained from a meteorological station at a nearby location: La Bonaigua, 6.3 km away from the study site and at a similar altitude, run by the Meteorological Service of Catalonia, www.meteo.cat, accessed in May 2014).

Experimental design

In July 2010 we established 30 plots of 1.1 m^2 combining a stand type and a warming treatment. We selected 10 pure stands of *V. myrtillus* (M stands), 10 mixed stands of *V. myrtillus* and *V. uligiosum* subsp. *microphyllum* (U stands), and 10 mixed stands of *V. myrtillus* and *Rhododendron ferrugineum* (R stands). In each stand, shrubs were the dominant species (Table S1), but other grasses and forbs were also present. Amongst them, the most abundant species were *Festuca eskia*, *Festuca nigrescens*, *Nardus stricta*, *Trifolium alpinum*, *Phleum alpinum* and *Meum athamanticum*. The distance between two plots ranged from one to a few metres (< 20 m), always ensuring that the studied patches were independent from each other. Soil organic matter and organic C concentrations, pH and colour (according to Munsell System) were similar across plots (Table S2). In half of the plots (i.e. 15 plots, five for each stand type) we placed an open-top chamber (OTC) made of transparent polycarbonate, similar to the model used in the International Tundra Experiment (ITEX; Marion et al. 1997). The other 15 plots served as ambient air temperature controls. The air temperature increase inside the OTCs in summer was 1.1 ºC, which we measured with temperature loggers (*iButton* 1-wire Thermochron, Embedded Data Systems, USA) placed at ground level in two plots of each stand type x warming combination during the growing season 2013 (recording every hour). The snow accumulation was high and homogenous along the study site and our phenological survey did not reveal substantial irregularities in the snowmelt pattern, even despite the presence of OTCs. Thus, we left the OTCs in place throughout the experiment.

Phenology and community composition

In 2011 we labelled six *V. myrtillus* ramets per plot, which we monitored during the growing seasons of 2011 and 2012 for a phenological survey. We recorded the following phenophases: winter state, bud swelling, bud bursting, leaf expansion, shoot elongation, vegetative state, leaf colour change, leaf shedding, leafless state and shoot winter colouring (brown-red coloration). We visited the plots *ca.* once a month starting after snowmelt until late Autumn, when ramets were leafless, and we recorded the presence of different phenophases in the six marked ramets. We assigned an ordinal numeric code to all phenophases and calculated the average numeric code per plot as the average score of the six ramets at each visit.

Plant community composition within the study plots was first recorded in 2011, by estimating the percentage cover of the main plant groups in each plot, i.e. shrubs and grasses. This was re-assessed in September 2013 before the end of the experiment (Table S1). Lichen and bryophyte cover was very low and was not recorded.

AG and BG biomass

On the 3rd September 2013 we harvested five *V. myrtillus* ramets per plot (not corresponding with those phenologically surveyed) plus five *V. uliginosum* ramets in U plots, avoiding sampling close to the edges of the OTCs. We also dug out their rhizomes (down to *ca.* 20 cm long) and the roots attached, and collected six soil cores of 12 cm length x 4 cm diameter in each plot (corresponding to 0-15 cm depth), which were kept in sealed plastic bags in a cool box until they arrived to the lab. Two of these cores were kept frozen and were used for BG biomass measurements at the plot scale. The rest were kept refrigerated at 4 ºC and were used for measurements of soil NO_3^- and NH_4^+ concentration and analyses of the functional diversity of the microbial community (two cores); and for rhizosphere C and N concentration and isotope composition analyses (two cores). Soil cores obtained for the same purpose from each plot were pooled together. Therefore, we had one composite soil sample per plot for each type of measurement.

Once in the lab, we measured the ramet height of both *Vaccinium* species and counted the scars left by the buds in each ramet to estimate their age. Then, we separated leaves, new shoots (i.e. shoots grown in 2013), rhizomes and roots of each species, and dried them at 60 ºC for 48 hours. Leaves and new shoots were weighed for AG biomass measurements and subsequently used for N and C concentration and isotope composition analyses. Rhizomes and roots were only used for N and C concentration and isotope composition analyses because BG biomass was measured at the plot scale on material obtained from the soil cores. We conducted BG biomass measurements referring to a specific soil volume to make comparisons between warming treatments and stand types possible. Soil cores for BG biomass measurements were sieved to separate rhizomes, coarse roots ($\geq$ 1 mm diameter) and fine roots (< 1 mm diameter). We dried them in the oven at 60 ºC for 48 h and weighed them for BG biomass analyses.

Carbon and nitrogen concentration and isotope composition

For the analyses of C and N concentration and isotope composition of leaves, new shoots, rhizomes and roots, we pooled together the material from all the harvested ramets of each plot for each plant part of each *Vaccinium* species. Then we ground the material and weighed *ca.* 1 mg subsamples in small tin capsules. The C and N concentrations of samples were determined using an Elemental Analyzer Flash 1112 (Carbo Erba, Milan). The C and N isotope composition of samples were determined using the Elemental Analyzer coupled to an IRMS Delta C isotope ratio mass spectrometer through a Conflo III Interface (Thermo-Finnigan, Germany). The results of C isotope analyses are reported in per thousand (‰) on the relative δ-scale as δ^{13}C, and refer to the international standard V-PDB (Vienna Pee Dee Belemnite) according to the following equation:

$$\delta^{13}\mathrm{C} = \left(\frac{R_{\mathrm{sample}}}{R_{\mathrm{standard}}}\right) - 1 \qquad \text{(Eq. 1)}$$

where R is the ^{13}C/^{12}C ratio.

N isotopic composition results are also expressed in δ notation (δ^{15}N) using international secondary standards of known ^{15}N/^{14}N ratios (IAEA N_1 and IAEA N_2 ammonium sulphate and IAEA NO_3 potassium nitrate) relative to N_2 in air:

$$\delta^{15}\mathrm{N} = \left(\frac{R_{\mathrm{sample}}}{R_{\mathrm{standard}}}\right) - 1 \qquad \text{(Eq. 2)}$$

where *R* is the $^{15}N/^{14}N$ ratio.

We used C and N concentrations to calculate C/N ratios of each plant part of the two *Vaccinium* species. We calculated total N pools for all *V. myrtillus* plant parts (for BG parts we only used data from pure stands, referred to soil volume) and for *V. uliginosum* AG plant parts. All EA-IRMS analyses were performed at the Scientific and Technological Centers (CCiT) of the University of Barcelona. The $\delta^{13}C$ of CO_2 of the air and the $\delta^{15}N$ of the bulk soil were analysed in 2013 and were *ca*. -10.91‰ and *ca*. 7.33 ‰, respectively (see Anadon-Rosell et al. 2016 for more information on air and soil sampling).

Soil inorganic N concentrations

NO_3^- concentrations were measured following the UV method described by Kaneko et al. (2010) by measuring the absorbance of KCl extracts from soils at 220 nm and 260 nm wavelengths. Soil NH_4^+ concentrations were measured by the conversion of ammonium into the intense blue indophenol complex (IPC) using salicylate, following the methods used by Kempers and Kox (1989).

Rhizospheric soil analyses and functional diversity of the microbial community

We carefully selected rhizomes and roots from the two soil cores collected for rhizosphere analyses and separated the soil that was attached using a small paint brush. We ground the soil and weighed *ca.* 3.5 mg subsamples in small tin capsules and analysed its C and N concentration and isotope composition following the same procedure as for plant tissues.

We assessed the impact of the warming treatment on the use of different C sources by soil microbial communities using Biolog EcoPlates (Insam 1997). Every plate had 96 wells containing 31 different C sources plus a blank well, in three replications. The rate of utilization of the C sources by microorganisms results in the increase of the optical density (OD_{590}) (Pohland and Owen 2009). We analysed the use of C by soil microbial communities in *V. myrtillus* pure stands only, using three replicates per warming treatment. Soils were sieved at 2 mm before extracting the bacterial community. Bacterial cells were extracted by mixing 10 g fresh soil with 95 ml of sterilized Milli-Q water inside 100 ml Erlenmeyers (see details in Muñiz et al. 2014). The mixture was magnetically shaken for 30 minutes, followed by one-hour rest. Afterwards, 10 ml of the soil suspension was put into 50 ml Falcon tubes and, after one-minute sonication, the tubes were centrifuged (1000 g, 10 minutes). 9.5 ml of the supernatant were separated and the remaining was resuspended after adding 9.5 ml of water. 47.5 ml of soil extract was obtained from each sample after five cycles of sonication-centrifugation. The extracts were kept at 4 °C for a few hours. Just before inoculating the Biolog plates, soil particles were removed by a low-speed centrifugation (500 g, 2 minutes). 150 µl of the soil extract were put into each well of the plates by a multipipete. All the laboratory material was sterile or it was previously autoclaved (121 °C, 20 minutes) and the operations were made inside a biological laminar flow chamber. The plates were incubated in the dark at 25 °C for 120 hours. The OD $_{590}$ of each well was measured just after the inoculation (at 0, 5, 72, 96, 101 and 120 h) using the Anthos 2010 microplate reader and ADAP 2.0 Software (Biochrom, Ltd. Cambridge Science Park, Cambridge, CB4 0FJ. England).

Statistical analyses

We tested the effects of warming and stand type on *V. myrtillus* phenology, ramet height and AG biomass using linear mixed effects models fitted with the restricted maximum likelihood estimation method (REML). We included warming and stand type as fixed factors and plot as a random factor to account for the multiple sampling within a plot. We used the same models for *V. uliginosum* variables, but in this case we only used warming as a fixed factor. To test the effects of warming and stand type on the C and N concentration and isotope composition of the different AG and BG tissues, BG biomass, soil NO_3^- and NH_4^+ concentration, rhizosphere C and N concentration and isotope composition we used simple linear model functions. We included ramet age as a covariate when it significantly contributed to improve model fits (which we tested with likelihood ratio tests) to account for possible age effects on our growth-related response variables. This was the case in the models for *V. myrtillus* height and AG biomass, plus the models for height, number of shoots and dry weight per shoot unit for *V. uliginosum*. We tested for significance with analysis of variance tests and graphically evaluated the assumptions of normality and homoscedasticity of residuals (Zuur et al. 2009). We double-checked with Shapiro and Bartlett tests when the visual evaluation of graphs was difficult. We log-transformed data when necessary to satisfy these assumptions. Moreover, when homoscedasticity of residuals was not met, we used the varIdent structure (Zuur et al. 2009; Pinheiro et al. 2016) to account for the heterogeneity of variances among factor levels. In *V. uliginosum* analyses, when both normality and homoscedasticity were not met, we used the non-parametric Wilcoxon test. We considered effects significant at $P < 0.05$ and trending towards significance at $0.05 > P < 0.10$ to account for the relatively low replication. When we found significant differences between stand types, we run Tukey HSD post hoc tests to determine those factor levels that differed significantly. We performed all the analyses with R 3.1.2 (R Core Team, 2015). For linear mixed effects models we used the *nlme* package (Pinheiro et al. 2016); for graphical evaluation of model assumptions we used the *lattice* package (Sarkar 2008); and for multiple comparisons we used the *multcomp* package on linear mixed effects models (Hothorn et al. 2008) and the *agricolae* package on simple linear models (de Mendiburu 2010).

The microbial activity of each microplate was expressed as the average well-colour development (AWCD) and was determined as previously described (Garland and Mills 1991) as follows:

$$\mathrm{AWCD} = \sum ODi / 31 \qquad \text{(Eq. 3)}$$

where OD_i is the optical density value from each well at any given time. The AWCD curves were adjusted to a log-logistic 4-parameters model using R Software. This was done first for each replicate and also for the warming treatment levels, each including all replicates (in this case with the raw data of OD_{590}). Then curve parameters (curve slope -slop-, maximal degradative capacity -max- and time to reach half of the slope -TM50-) were compared using the *CompParm* function in *drc* package (Ritz and Streibig 2005): the null hypothesis was that the ratio between parameters equalled 1. The ratio was obtained by dividing the same parameters from each curve by the other parameters; if the ratio significantly differed from 1, the null hypothesis was rejected, meaning that values were significantly different ($P < 0.05$). P-values were adjusted using Bonferroni correction for multiple T-tests.

Results

Phenology

Warming advanced early-season vegetative phenology through an earlier onset of bud burst and leaf expansion (Fig. S1). On the 21st May 2011 (day of year, DOY, 141), *V. myrtillus* ramets in unwarmed plots were at the bud swelling phase, whereas in warmed plots had already started bursting ($F_{1,24}$ = 3.92, P = 0.059). In 2012, *V. myrtillus* ramets in warmed plots were expanding their leaves on the 14th June (DOY 166), whereas ramets in unwarmed plots were still in the bud burst phenophase ($F_{1,24}$ = 6.59, P = 0.017). Monitoring later in the season for both years did not show any other significant differences between warming treatments (see Fig. S1 for visit dates). We only found significant differences between stand types (regardless of the warming treatment) in September 2011, when ramets in M stands were already shedding their leaves whereas ramets in the other two stand types had just started changing their colour prior to leaf shedding ($F_{2,24}$ = 9.31, P = 0.001). This advancement in senescence in M stands with respect to the other stand types was especially obvious in warmed plots (the interaction stand type x warming trended towards significance, $F_{2,24}$ = 3.13, P = 0.062).

Age and AG biomass of Vaccinium *species*

Our age analyses confirmed that there were no differences in *V. myrtillus* ramet age between warming treatments ($F_{1,24}$ = 0.16, P = 0.696) nor between stand types ($F_{2,24}$ = 1.89, P = 0.173). Likewise *V. uliginosum* did not show differences in ramet age between warming treatments ($F_{1,6}$ = 0.01, P = 0.930).

After four years of warming, *V. myrtillus* ramets were 15% taller in warmed plots than in unwarmed plots. There were no differences in ramet height between stand types or an interaction between warming and stand type (Table 1). Similarly, there was no warming effect on *V. uliginosum* height ($F_{1,6}$ = 0.08, P = 0.784).

Vaccinium myrtillus leaf biomass per ramet did not differ between warming treatments (Table 1, Fig. 1a) but new shoot biomass was higher under warming than in control plots (Fig. 1b). The total above-ground biomass per ramet was also higher in warmed plots than in unwarmed plots (Table 1, Fig. 1c). There were no differences between stand types or a stand type x warming interaction for *V. myrtillus* AG biomass (Table 1). There were no differences between warming treatments in terms of *V. uliginosum* leaf biomass ($F_{1,6}$ = 2.77, P = 0.147), new shoot biomass ($F_{1,6}$ = 0.04, P = 0.849) or total AG biomass ($F_{1,6}$ = 0.39, P = 0.554, Fig. S2), but we found contrasting effects of warming on the dry weight per shoot and the number of new shoots. Dry weight per shoot in *V. uliginosum* was higher inside the OTCs than in control plots ($F_{1,6}$ = 6.42, P = 0.044), whereas the number of new shoots was higher in ramets from unwarmed plots ($F_{1,6}$ = 14.81, P = 0.009).

Vaccinium myrtillus *BG biomass*

There were no effects of warming on *V. myrtillus* BG biomass (Fig. 1d, e, f). We only found differences in rhizome and coarse root biomass between stand types. R stands showed lower rhizome biomass per soil volume than in the other two stand types ($F_{2,24}$ = 6.93, P = 0.004, Fig. 1d). U stands showed a trend towards significantly greater coarse root biomass than M stands ($F_{2,19}$ = 3.04, P = 0.071, Fig. 1e). Fine root biomass did not differ between stand types ($F_{2,24}$ = 0.41, P = 0.667, Fig. 1f). We did not find any warming x stand type interaction for any of the BG plant parts analysed (Table S3).

C and N concentration and isotope composition of AG and BG plant fractions

C concentration in *V. myrtillus* organs was similar across warming treatments and stand types for leaves, shoots and roots. Rhizomes, however, had greater C concentration under warming than in control plots (Table 2, Fig. 2), which was not related to any rhizome biomass increase under warming (see above). C concentration values of *V. uliginosum* new shoots, rhizomes and roots did not show any response to warming, but there was a trend towards a significantly positive effect of warming on leaf C concentration (Table 3, Fig. 3).

The δ^{13}C of *V. myrtillus* and *V. uliginosum* tissues did not differ between warming treatments (Fig. 2, 3) but we found significant differences in the δ^{13}C of *V. myrtillus* tissues between stand types. *Vaccinium myrtillus* δ^{13}C was lower in R stands than in the other two stand types for leaves (only trending towards significance), shoots and rhizomes. There were no significant differences between stand types for the δ^{13}C composition of roots (Table 2, Fig. 2), or any warming x stand type interaction.

There was no warming effect on the N concentration and δ^{15}N of any of the *V. myrtillus* organs, and only a very marginal trend towards significance of the interaction between warming and stand type in the N concentration of *V. myrtillus* rhizomes, which was higher in control plots than in warmed plots in U stands (Table 2, Fig. 2). However, we found significant differences between stand types. Leaf N concentration was higher in R stands than in U stands, but this was not the case for any of the other plant organs. Leaf and shoot δ^{15}N values were higher in M stands than in the other two stand types. Finally, rhizome δ^{15}N values were also higher in M stands than in the other two stand types, but only significantly higher than in R stands (Table 2, Fig. 2). The N pool in *Vaccinium myrtillus* rhizomes (0.037 ± 0.006 mg/cm^3) and roots (0.010 ± 0.002 mg/cm^3) did not differ between warming treatments ($F_{1,7} = 0.51$, $P = 0.497$; $F_{1,8} = 0.60$, $P = 0.462$, respectively). Leaf N pools did not differ between warming treatments ($F_{1,24} = 2.21$, $P = 0.150$) or stand types ($F_{2,24} = 1.34$, $P = 0.282$) (average across warming and stand type treatments of 9.16 ± 0.65 mg). The N pool in *V. myrtillus* new shoots was higher in warmed (5.46 ± 0.62 mg) than in unwarmed plots (3.72 ± 0.38 mg) ($F_{1,24} = 5.82$, $P = 0.024$), but did not differ between stand types ($F_{2,24} = 0.93$, $P = 0.408$). We did not find any warming effect on C/N ratios, only a trend towards significance for rhizomes in U stands, which showed higher values under warming than in unwarmed plots (Table 2, Fig. S3). We only found significant differences in C/N ratios between stand types in leaves, which showed higher values in U stands than in the other two (Table 2, Fig S3). New shoots and roots did not show significant differences in their C/N ratios for any of the treatments.

Vaccinium uliginosum shoots showed significantly lower N concentrations under warming than in unwarmed plots. This seemed associated with an increase in leaf N concentrations under warming (although the latter was not significant; Table 3). δ^{15}N values did not differ significantly between warming treatments (Table 3, Fig. 3). Leaf and new shoot N pools in *V. uliginosum* did not differ between warming treatments either ($F_{1,6} = 1.74$, $P = 0.235$; $F_{1,6} = 0.04$, $P = 0.843$, respectively). The total amount of N in leaves was 9.25 ± 0.78 mg and in new shoots 1.92 ± 0.23 mg (averaged across warming treatments). C/N ratios did not differ significantly between warming treatments in leaves (24.58 ± 0.74, $F_{1,6} = 1.59$, $P = 0.254$), rhizomes (93.12 ± 6.30, $F_{1,6} = 0.03$, $P = 0.874$) or roots (53.87 ± 1.96, $F_{1,6} = 0.90$, $P = 0.379$) (averages across warming treatments shown). However, in new shoots C/N values were higher under warming (52.37 ± 0.95) than in unwarmed plots (46.28 ± 1.84; $F_{1,6} = 8.65$, $P = 0.026$).

Soil inorganic N concentrations, rhizosphere C and N and functionality of the soil microbial community

Soil NO_3^- concentration decreased by 36% in warmed plots compared with unwarmed plots ($F_{1,24}$ = 5.87, P = 0.023, Fig. 4a), but the NH_4^+ concentration remained similar between warming treatments (F = 0.45, P = 0.508, Fig. 4b). As a consequence, the NO_3^-/ NH_4^+ ratio decreased by 27% under warming with respect to control conditions. There was no difference between stand types or any interaction between warming and stand type for any of the two N forms analysed.

The rhizosphere C/N ratio did not differ between warming treatments. However, it differed between stand types, as it was higher in U stands than in the other two ($F_{2,24}$ = 7.99, P = 0.002, Fig. S4). Both rhizosphere soil C and N concentration were significantly higher in U stands than in R and M stands ($F_{2,24}$ = 5.81, P = 0.009 and $F_{2,24}$ = 3.64, P = 0.042, respectively), but the difference in the C concentration was greater than the difference in N (data not shown). There was no significant warming x stand type interaction on the rhizosphere C/N ratio ($F_{2,24}$ = 0.89, P = 0.422), but the high dispersion in the data could have masked possible differences between warming treatments in U stands. Neither warming nor stand type or their interaction had any effects on rhizospheric soil $\delta^{13}C$ and $\delta^{15}N$ values ($P > 0.28$).

The warming treatment did not change the ability of the microbial communities of *V. myrtillus* M stands to degrade the different sources of C present in the Ecoplates (Fig. S5 and S6), indicating that the exposure to 1.1 ºC warmer temperatures during four years was not enough to result in significant changes in the soil C cycling by microbia.

Discussion

Vaccinium myrtillus slightly benefitted from increased temperatures regardless of neighbourhood composition. Thus, our results evidence that four years of passive warming did not lead to changes in the interaction between *V. myrtillus* and its neighbour species at our study site. Although coexistence theory predicts that intraspecific competition should be stronger than interspecific competition (Chesson 2000), we did not find evidence of stronger competition in pure than in mixed stands nor that this changed with warming, even when the percentage cover of *V. myrtillus* ramets in pure stands was higher than in mixed stands (and also ramet density, which was measured in 2010 in similar plots at the same study site; Table S1). A previous study at the same area also found that the performance of *V.*

myrtillus was similar across stand types, indicating that ramets did not perform worse in pure stands than in mixtures (Anadon-Rosell et al. 2016). Furthermore, other studies have reported that intraspecific facilitation can be more important than competition at moderate to high levels of stress (Chu et al. 2008; Fajardo and McIntire 2011).

The lack of warming effects on species interactions in our study contrasts with previous studies in which the interaction between plant species shifted when temperatures changed (both naturally and experimentally). Dormann et al. (2004) found that the interaction between the rush *Luzula confusa* and the deciduous shrub *Salix polaris* changed with warming in favour of *S. polaris* in Svalbard. In a removal experiment in Finse, Norway, Klanderud and Totland (2005) found that the removal of the neighbour species negatively affected *Thalictrum alpinum* in unwarmed plots, but not inside OTCs, indicating that warming affected the interaction between these species. Callaway et al. (2002) also reported evidence of a shift from facilitation at higher elevation sites to competition at lower elevations when removing neighbours of target individuals at different mountain sites across the world. In addition, a study in seminatural grasslands in southern Norway found increased competitive interactions with increasing temperature across precipitation and temperature gradients (Olsen et al. 2016). Most of these studies consisted of removal experiments, which provide very important ecological and functional information about the community and species studied, and allow to test the effect of species composition on the community response to the environment (see review by Díaz et al. 2003). However, despite their numerous advantages and outcomes, removal experiments cannot avoid the disturbance caused by the extraction of selected species. In contrast, our approach was based on naturally established populations and species and allowed us to assess their natural responses to warming. According to our results, species interactions seem less responsive to warming when studied under natural conditions and within the natural distributions of the study species. While the removal experiments mentioned above captured changes in species interactions after shorter periods of warming than our experiment, four years of passive warming were not sufficient to cause changes in species interactions at our study site.

The AG biomass increase of *V. myrtillus* inside the OTCs could be the result of a longer growing period caused by the advancement of its early-vegetative phenology. A previous study on the same species in the Swiss Alps showed that its increased growth after six years of soil warming with heating cables was not related to a longer growing period (Anadon-Rosell et al. 2014). The above-ground phenology of ramets could be more affected by warming through OTCs than by soil warming, since air temperature at canopy level may be higher inside the OTC. However, other factors related to warming but not directly linked to a longer growing season could have also influenced *V. myrtillus* growth in our study, such as direct warming effects on photosynthetic rates (Heskel et al. 2013; Fu et al. 2015) or through the stimulation of N uptake rates, which would be supported by the lower soil NO_3^- values in warmed plots at our study (see below). In addition, although OTCs are a robust tool for the study of warming effects on plant species (Hollister and Webber 2000; see review by Bokhorst et al. 2013), they might potentially cause confounding effects on microclimate variables that could have influenced the response of our study species to the warming treatment, such as modifications in wind patterns around the study plants (DeBoek et al. 2012) and changes in soil moisture and vapour pressure deficit (Marion et al. 1997; Piiki et al. 2008).

In our study, the BG biomass of *V. myrtillus* did not change with warming, and this was consistent across stand types. Thus, the AG growth stimulation under warming did not result in increased BG growth, indicating an uncoupling between AG and BG responses to warming. This could potentially be related to different phenological responses to increased temperatures. In fact, an uncoupling between AG and BG phenology has been reported along an Arctic elevation gradient (Blume-Berry et al. 2016). Another explanation could be that OTCs mainly increase ground-level and air temperature. However, they have been found to slightly increase soil temperature at 5 cm depth (Hollister et al. 2006) and even at 10 cm in steppe ecosystems in Northern Mongolia (Sharkhuu et al. 2013). Hollister and Flaherty (2010) found a BG biomass increase in *Salix rotundifolia* at the Alaskan tundra after 3–4 years of warming with OTCs, but Shaver et al. (1998) found no BG biomass increase after 6–9 years of passive warming in another Alaskan wet sedge tundra site, indicating contrasting BG responses to warming depending on the study site and community composition. *Vaccinium myrtillus* can expand its rhizomes laterally several metres below-ground (Flower-Ellis, 1971); therefore our warming treatment might have not captured the potential response of a whole functional unit to warming, or a possible transfer of assimilates from AG parts might have been diluted by the complex BG network of this species. The lack of differences between warming treatments in the rhizosphere C/N ratio and in the soil microbial C source use suggests that the degree of warming applied in our study was not enough to induce significant BG changes. Moreover, the similar substrate utilization by the microbial community between warming treatments indicates that the soil C pools were not altered by the increased temperature (Rinnan et al. 2009). Numerous studies on the effect of temperature on the composition and functioning of soil microbial communities have led to contrasting results (see review by Classen et al. 2015). Temperature shifts of as much as 10 ºC did not significantly alter the physiological functioning of the microbiota of humic soils (Pettersson and Bååth 2003). However, a temperature increase of 5 ºC in temperate forests resulted in a significant alteration of soil microbial communities (DeAngelis et al. 2015), and an increase of 4 ºC in a warming experiment at the Swiss treeline led to changes in the microbial substrate use (Streit et al. 2014). All these contrasting results indicate that warming effects on microbial metabolism and BG processes may be mediated through other biotic and abiotic factors (see also Christiansen et al. 2017), and certainly deserve further study.

Vaccinium uliginosum has been shown to be less plastic in response to warming than *V. myrtillus* (Richardson et al. 2002; Kudo and Suzuki 2003; Anadon-Rosell et al. 2014). This can be attributed to the better adaptation of *V. myrtillus* to higher temperatures, which is evidenced by the fact that it grows at lower altitudes than *V. uliginosum* (Bolòs et al. 2005). Although the dry weight of new individual shoots of *V. uliginosum* increased with warming, the number of shoots decreased, probably as a trade-off, which led to an overall lack of AG biomass response to warming in this species. In fact, only the leaf C concentration of *V. uliginosum* increased slightly with warming, and was not accompanied by any other changes in the shrub performance. Our study provides evidence that although *V. myrtillus* is more responsive to warming than *V. uliginosum*, it does not benefit more from warming when it grows in mixtures than when it grows in pure stands.

The slightly lower N concentration in *V. myrtillus* rhizomes in warmed plots than in unwarmed plots when coexisting with *V. uliginosum* suggests that there could be an increase in competition for N with warming. In fact, competition for N was found in mixed stands of the two *Vaccinium* species under natural conditions in a previous study at the same site (Anadon-Rosell et al. 2016), and is supported by the higher rhizosphere and leaf C/N ratios in mixed stands of these two species than in pure stands found in this study. Moreover, the higher $\delta^{15}N$ values in pure stands than in mixed stands, which might be explained by lower N uptake through mycorrhiza or larger ecosystem losses of ^{15}N-depleted N (leaving an enriched remaining pool), are indicative of larger N availability (Craine et al. 2009). A study in the Swiss Alps found a positive response to warming in *V. uliginosum* leaf N concentration but only a short-term positive response in *V. myrtillus* (Dawes et al. 2011). On the other hand, in the Swedish Lapland, *V. myrtillus* increased leaf N concentration in response to warming whereas the opposite was found for *V. uliginosum* (Richardson et al. 2002). However, these studies did not test warming effects on interspecific interactions. Our study suggests that although warming may increase the competition for N between *V. myrtillus* and *V. uliginosum*, this does not outweigh the positive growth response of *V. myrtillus* to warming.

A meta-analysis of experimental warming effects on N pools in terrestrial ecosystems showed that warming increased N mineralization rates and N pools across different ecosystem types (Bai et al. 2013). However, in our experiment soil NO_3^- decreased with warming (regardless of the stand type). This could be due to greater NO_3^- uptake rates promoted by warming, since temperature is a modulator of plant N assimilation (Laine et al. 1994; Volder et al. 2000). The lack of an increase in the N concentration of *V. myrtillus* tissues could be due to a dilution effect caused by the greater biomass, which is supported by the higher total N pool found in new shoots under warming. Additionally, other species (especially grasses, due to their abundance) could have increased their NO_3^- assimilation under warming, which was not assessed in this study. Another explanation for the reduced soil NO_3^- concentrations in the OTCs may be earlier consumption of NO_3^- through an advanced root phenology promoted by warming (Sullivan and Welker 2005; Nord and Lynch 2009). In the Finnish tundra, Rinnan et al. (2009) detected no increase in soil N concentration with warming either, but there was a decrease in soil NH_4^+ concentration inside the OTCs. The authors argued that this reduction could reflect the increased efficiency of N uptake with warming. The differing responses in the N form between that study and ours might reflect the differential use of specific N forms at different sites with different community composition, or a greater availability of NO_3^- than NH_4^+ at our study site.

In conclusion, four years of experimental warming had no effect on the interaction between *V. myrtillus* and *V. uliginosum* or *R. ferrugineum*. *Vaccinium myrtillus* showed a positive AG biomass response to warming regardless of the neighbouring species, but no BG responses were found. Although warming seemed to increase the competition for N between the two *Vaccinium* species, their overall performance was not affected. This study shows that species interactions are not altered by warming at this treeline site and, thus, the performance of these populations will probably not change due to mild warming in the near future.

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Tables

Table 1. Results of ANOVA for effects of warming and stand type on *Vaccinium myrtillus* height and above-ground (AG) biomass parameters at the ramet scale

AG Variable	Factor/covariable	*df*	*F*	*P*
Height	Age	1, 118	26.47	<0.001
	Warming	1, 24	5.33	0.030
	Stand type	2, 24	1.89	0.173
	Stand type x warming	2, 24	0.16	0.857
Leaf biomass	Age	1, 118	12.00	0.001
	Warming	1, 24	2.38	0.136
	Stand type	2, 24	1.49	0.246
	Stand type x warming	2, 24	1.20	0.320
New shoots biomass	Age	1, 118	8.68	0.004
	Warming	1, 24	5.02	0.035
	Stand type	2, 24	1.38	0.271
	Stand type x warming	2, 24	0.37	0.693
Total AG biomass	Age	1, 118	32.41	<0.001
	Warming	1, 24	4.74	0.040
	Stand type	2, 24	1.77	0.193
	Stand type x warming	2, 24	0.16	0.857
No. of shoots	Age	1, 114	23.45	<0.001
	Warming	1, 23	0.26	0.613
	Stand type	2, 23	0.84	0.443
	Stand type x warming	2, 23	0.83	0.450
Dry weight/shoot	Age	1, 114	2.87	0.093
	Warming	1, 23	3.88	0.061
	Stand type	2, 23	2.24	0.129
	Stand type x warming	2, 23	0.55	0.584

Table 2. Results of ANOVA testing the effects of warming (W), stand type (ST) and their interaction (W x ST) on the C and N concentration and isotope composition ($\delta^{13}C$, $\delta^{15}N$) and C/N ratio of *Vaccinium myrtillus* leaves, new shoots, rhizomes and roots. *F*-values and *P*-values (in parentheses) are given. Between-groups degrees of freedom were 1 for W, 2 for ST and 2 for ST x W. Within-groups degrees of freedom were 24, except for root N concentration and C/N (22), rhizome C and N concentration (22), root $\delta^{15}N$ (23) and rhizome C/N (21)

Organ	Variable	W	ST	W x ST
Leaves	C concentration	0.93 (0.344)	1.51 (0.242)	1.57 (0.228)
	N concentration	0.02 (0.884)	4.93 (0.016)	0.07 (0.931)
	$\delta^{13}C$	0.90 (0.352)	2.72 (0.086)	0.85 (0.441)
	$\delta^{15}N$	0.08 (0.780)	10.28 (0.001)	0.04 (0.960)
	C/N	0.06 (0.796)	3.74 (0.038)	0.23 (0.798)
New shoots	C concentration	1.68 (0.207)	0.94 (0.404)	0.94 (0.404)
	N concentration	0.07 (0.793)	0.63 (0.540)	0.77 (0.472)
	$\delta^{13}C$	0.07 (0.794)	8.16 (0.002)	1.85 (0.179)
	$\delta^{15}N$	0.33 (0.571)	9.39 (0.001)	0.00 (1.000)
	C/N	0.70 (0.410)	0.84 (0.444)	0.23 (0.794)
Rhizomes	C concentration	5.71 (0.026)	0.33 (0.723)	0.7 (0.509)
	N concentration	0.05 (0.829)	0.46 (0.637)	2.57 (0.099)
	$\delta^{13}C$	0.42 (0.522)	8.78 (0.001)	0.03 (0.972)
	$\delta^{15}N$	0.02 (0.884)	6.53 (0.005)	0.08 (0.921)
	C/N	2.71 (0.114)	0.26 (0.775)	2.70 (0.091)
Roots	C concentration	0.21 (0.653)	0.43 (0.656)	0.56 (0.578)
	N concentration	1.69 (0.207)	0.62 (0.545)	1.19 (0.323)
	$\delta^{13}C$	1.59 (0.218)	0.15 (0.860)	2.53 (0.101)
	$\delta^{15}N$	0.21 (0.650)	2.04 (0.153)	0.19 (0.826)
	C/N	1.58 (0.222)	0.31 (0.739)	0.38 (0.688)

Table 3. Results of ANOVA or Wilcoxon tests for the effects of warming on the C and N concentration and isotope composition ($\delta^{13}C$, $\delta^{15}N$) of *Vaccinium uliginosum* leaves, new shoots, rhizomes and roots

Organ	Parameter	*df*	*F*/*W*	*P*
Leaves	C concentration	1, 6	3.89	0.096
	N concentration	1, 6	2.57	0.160
	$\delta^{13}C$	1, 6	0.01	0.934
	$\delta^{15}N$	-	$W = 6$	0.686
New shoots	C concentration	-	$W = 6$	0.686
	N concentration	1, 6	13.91	0.010
	$\delta^{13}C$	1, 6	0.03	0.871
	$\delta^{15}N$	1, 6	0.35	0.575
Rhizomes	C concentration	1, 6	0.99	0.357
	N concentration	-	$W = 8$	1.000
	$\delta^{13}C$	-	$W = 8$	1.000
	$\delta^{15}N$	-	$W = 8$	1.000
Roots	C concentration	1, 6	0.00	0.997
	N concentration	1, 6	0.93	0.373
	$\delta^{13}C$	1, 6	1.08	0.339
	$\delta^{15}N$	1, 6	3.86	0.097

Figure legends

Figure 1. Effects of stand types (ST) and warming treatment (W) on a-c) aboveground and d-f) belowground biomass (means + 1 SE; n = 5) of *Vaccinium myrtillus*. Stand types included: *V. myrtillus* pure stands (M); *V. myrtillus* mixed with *R. ferrugineum* stands (R) and *V. myrtillus* mixed with *V. uliginosum* stands (U). Note that BG biomass data are reported as unit volume of soil at the plot scale. Symbols indicate significance levels: († 0.1 > P > 0.05; * 0.05 > P > 0.01; ** P < 0.01)

Figure 2. Effects of stand types (ST) and warming treatment on C and N concentrations and isotope compositions ($\delta^{13}C$ and $\delta^{15}N$) of leaves, new shoots, rhizomes and roots (mean ±1 SE; n = 5) of *V. myrtillus* organs in different stand types (ST) and warming (W) treatments. Stand types included: *V. myrtillus* pure stands (M); *V. myrtillus* mixed with *R. ferrugineum* stands (R) and *V. myrtillus* mixed with *V. uliginosum* stands (U). Symbols indicate significance levels: († 0.1 > P > 0.05; * 0.05 > P > 0.01; ** P < 0.01). Y-axis values for nitrogen concentration in roots differ from the rest of the tissues, and are indicated at the right side of the panel

Figure 3. Effects of warming treatment on a) C and b) N concentrations and c) $\delta^{13}C$ and d) $\delta^{15}N$ of *V. uliginosum* organs (leaves, shoots, rhizomes and roots; mean ±1 SE; n = 4). Symbols indicate significance levels: († 0.1 > P > 0.05; * 0.05 > P > 0.01; ** P < 0.01)

Figure 4. Soil a) nitrate (NO_3^-) and b) ammonia (NH_4^+) concentration in our study plots in different stand types (ST) and warming treatments in September 2013 (W; n = 5, means + 1 SE are shown). Stand types included: *V. myrtillus* pure stands (M); *V. myrtillus* mixed with *R. ferrugineum* stands (R) and *V. myrtillus* mixed with *V. uliginosum* stands (U). Asterisks (**) show significant differences at P < 0.01

Figure 1

Fig. 1

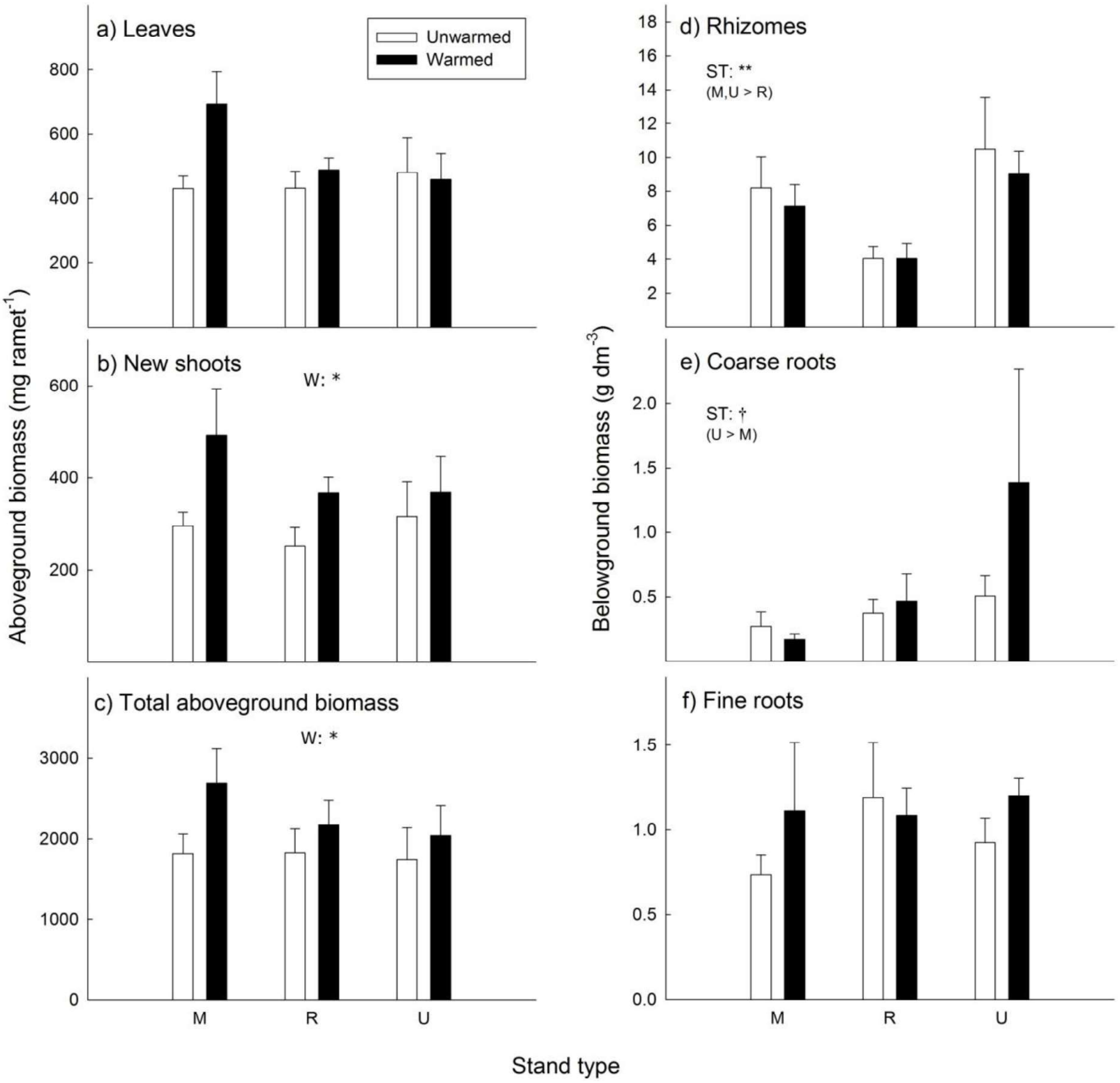

Figure 2

Fig. 2

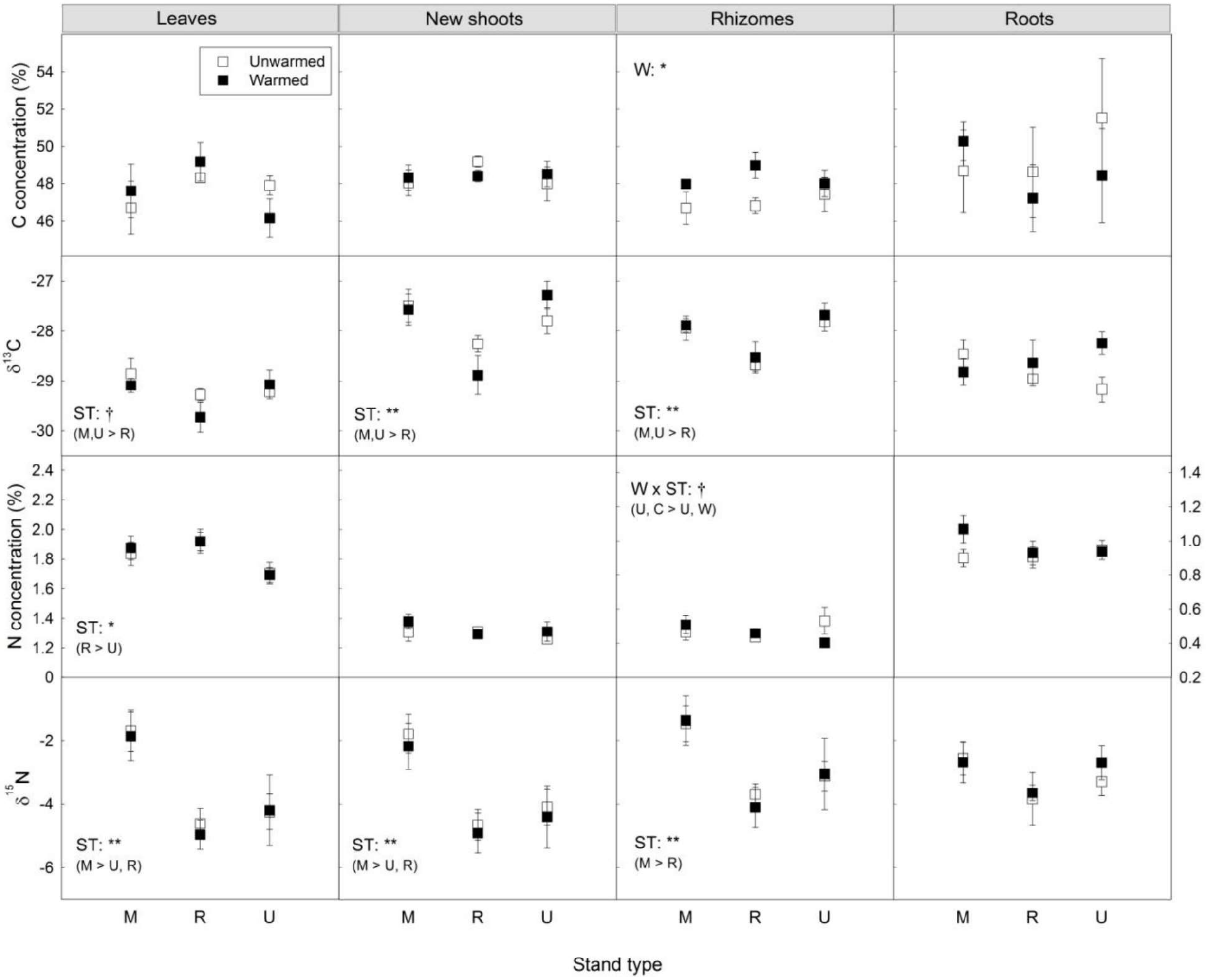

Figure 3

Fig. 3

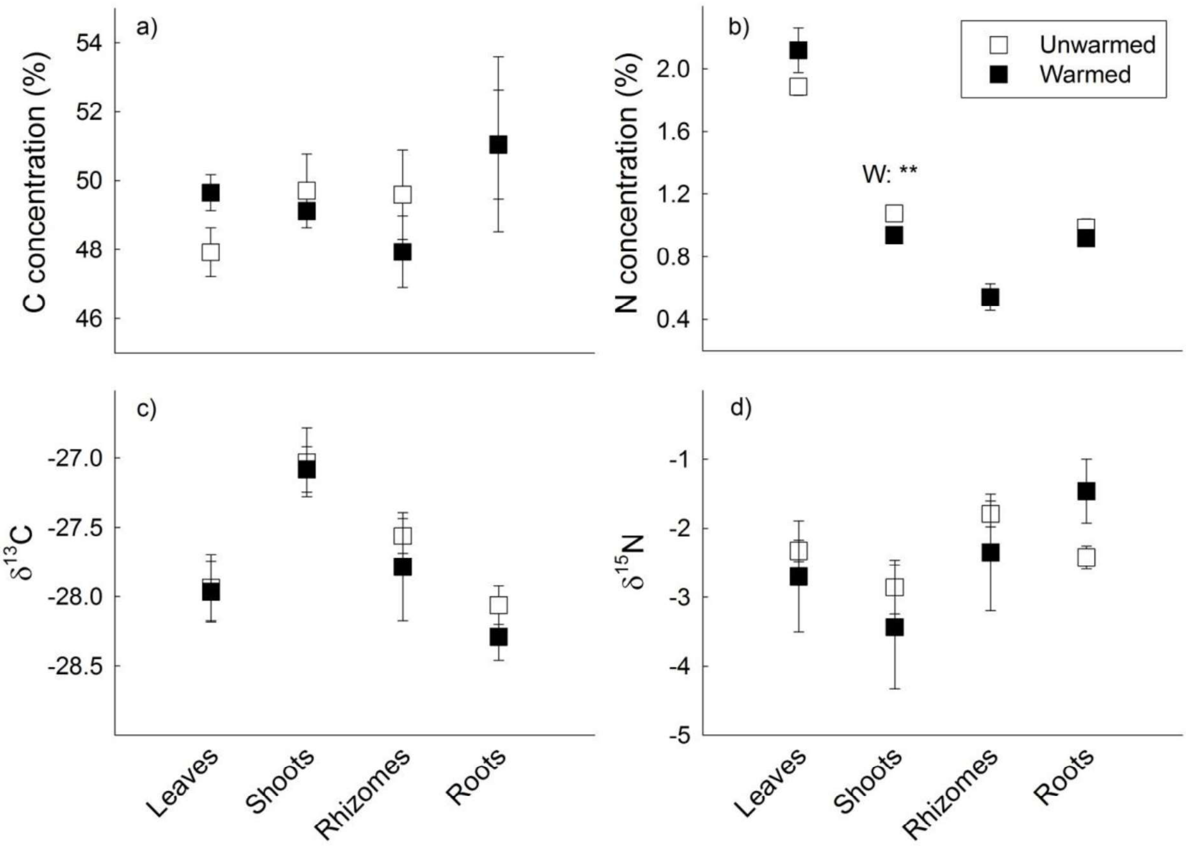

Figure 4

Fig. 4

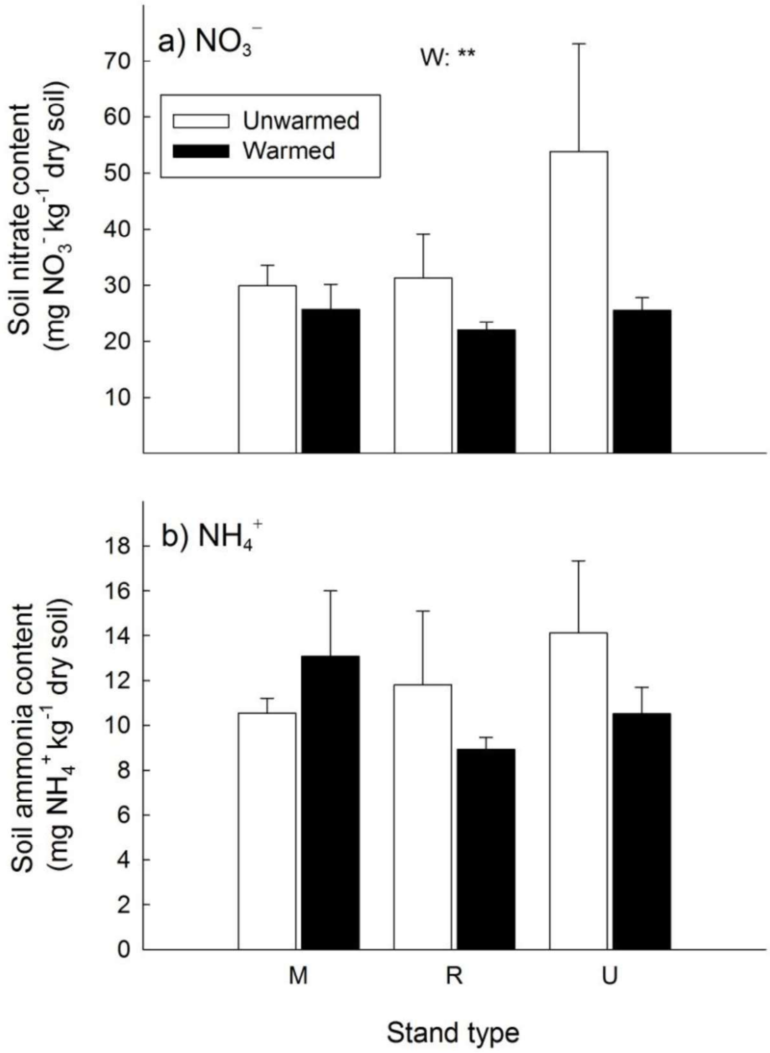

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Fig. 1

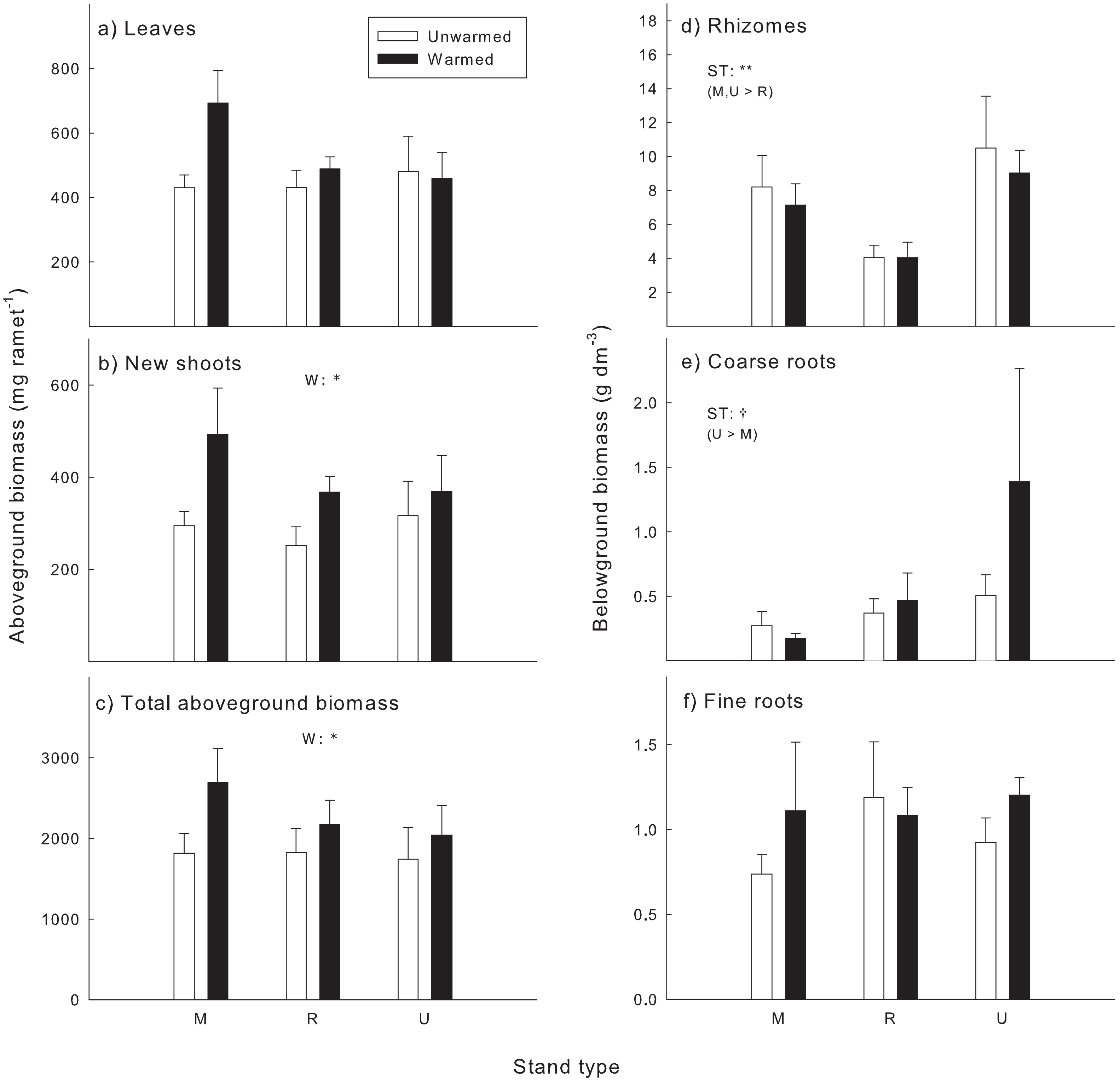

a) Leaves
Unwarmed
Warmed
800
600
400
200
Aboveground biomass (mg ramet^{-1})
b) New shoots
W: *
600
400
200
c) Total aboveground biomass
W: *
3000
2000
1000
0
M
R
U
d) Rhizomes
ST: **
(M,U > R)
18
16
14
12
10
8
6
4
2
Belowground biomass (g dm^{-3})
e) Coarse roots
ST: †
(U > M)
2.0
1.5
1.0
0.5
f) Fine roots
1.5
1.0
0.5
0.0
M
R
U
Stand type

Fig. 2

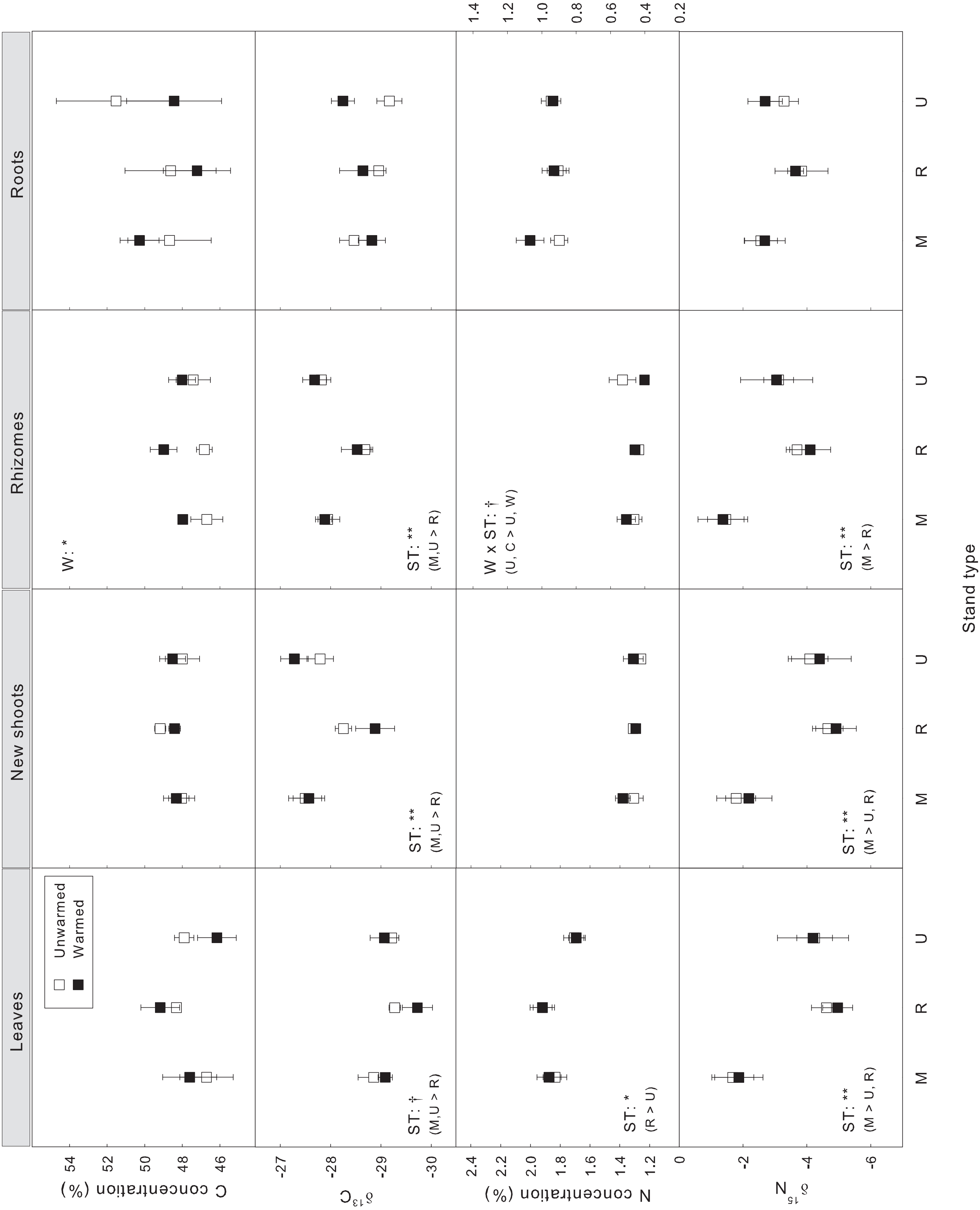

Leaves
New shoots
Rhizomes
Roots
□ Unwarmed
■ Warmed
C concentration (%)
54
52
50
48
46
δ13C
-27
-28
-29
-30
N concentration (%)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
δ15N
-2
-4
-6
W: *
ST: †
(M,U > R)
ST: **
(M,U > R)
ST: **
(M,U > R)
ST: *
(R > U)
W x ST: †
(U, C > U, W)
ST: **
(M > U, R)
ST: **
(M > U, R)
ST: **
(M > R)
M
R
U
Stand type

Figure 3

Fig. 3

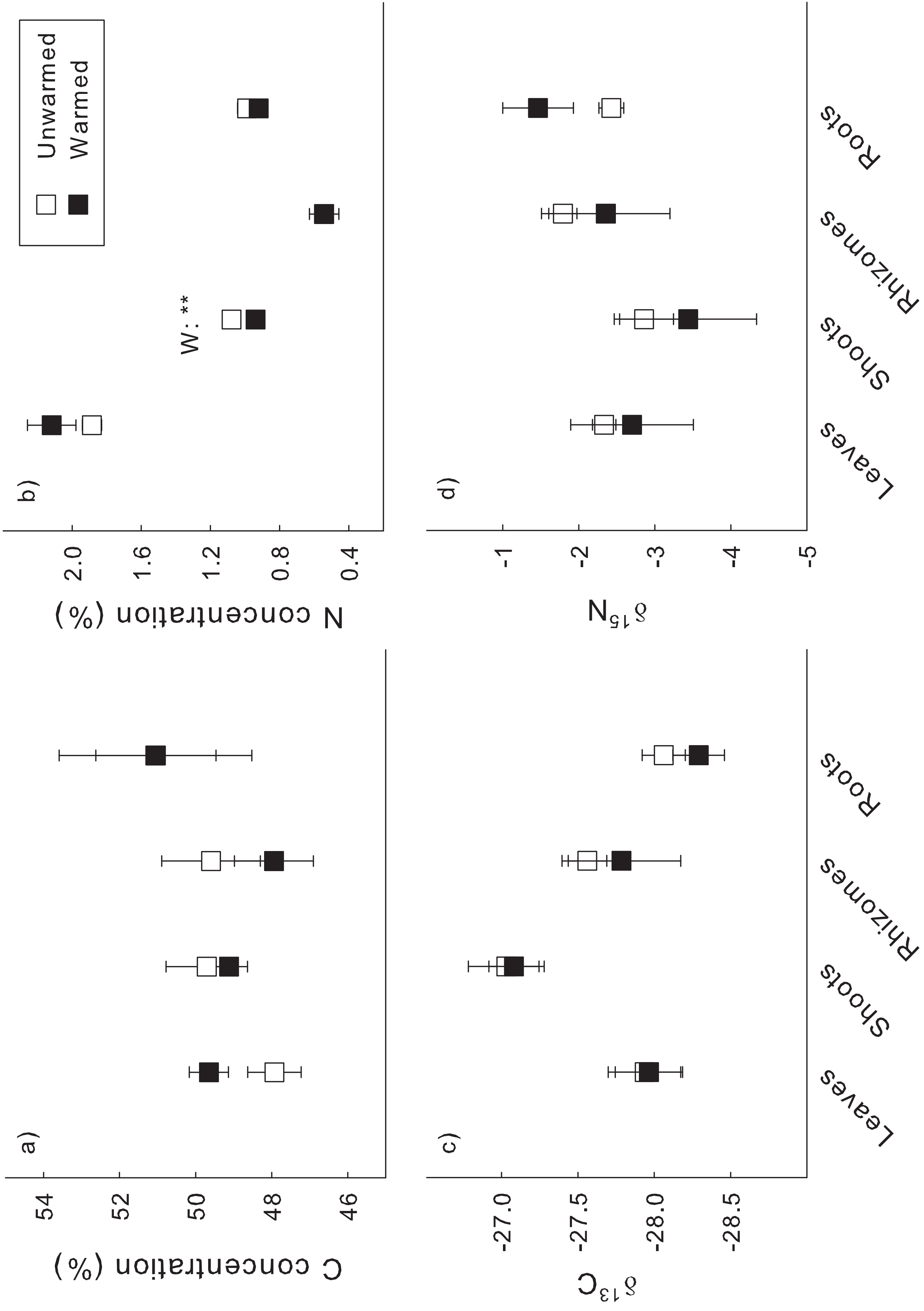
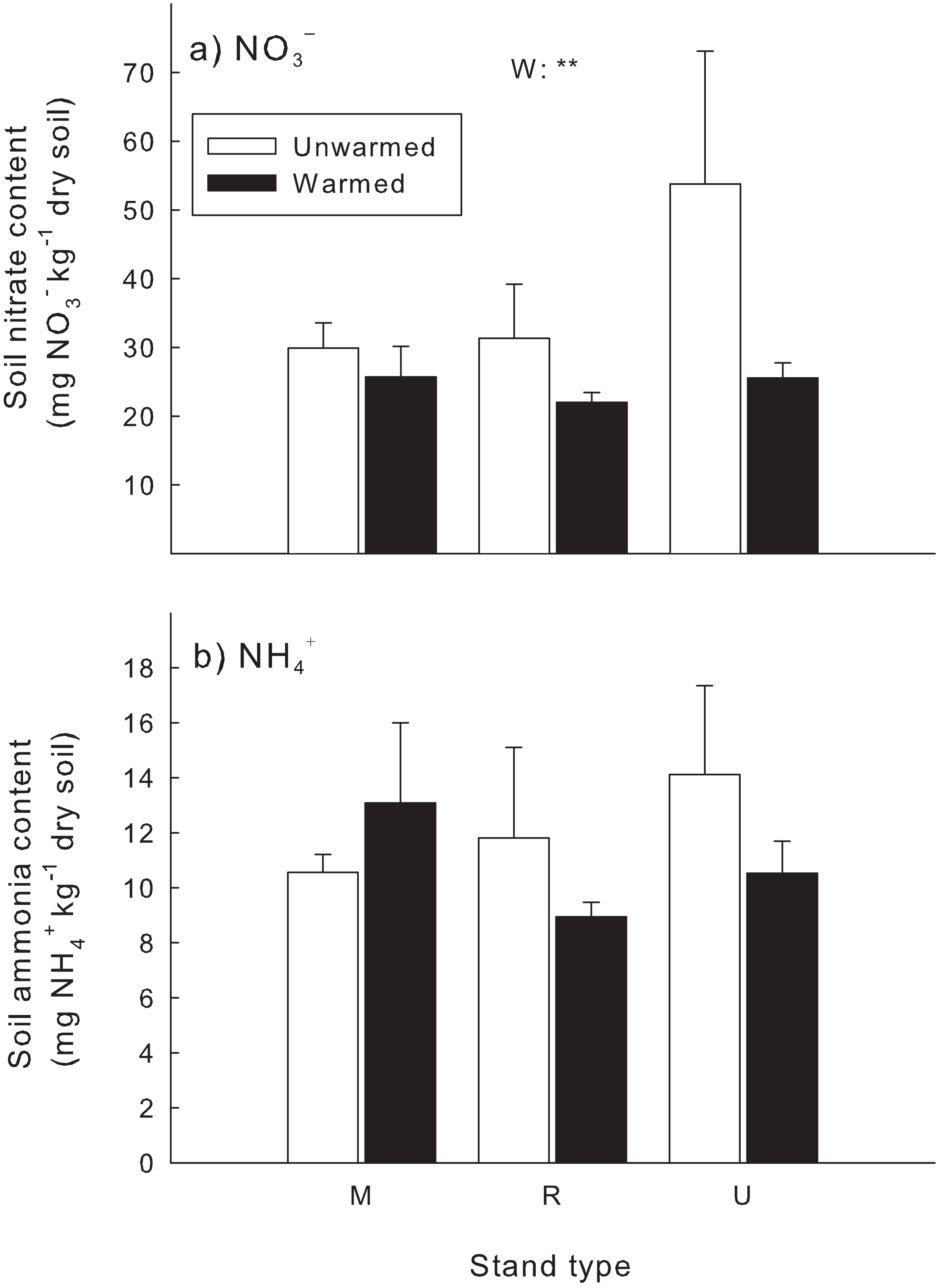


Fig. 4


a) NO_3^-
W: **
Unwarmed
Warmed
Soil nitrate content
(mg NO_3^- kg^{-1} dry soil)
70
60
50
40
30
20
10
b) NH_4^+
Soil ammonia content
(mg NH_4^+ kg^{-1} dry soil)
18
16
14
12
10
8
6
4
2
0
M
R
U
Stand type