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Warming alters competition for organic and inorganic nitrogen between co-existing grassland plant species

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Abstract

- Grass species may acquire different forms of nitrogen (N) to reduce competition for the same resources. Climate change influences the availability of soil N and is therefore likely to cause shifts in N forms acquired by plants, thereby affecting their competitive interactions.
- We investigated the effects of warming on the uptake of different N forms and competitive interactions of *Festuca ovina* and *Anthoxanthum odoratum* in a pot experiment. The plants were grown either in monocultures or mixture, and at ambient or elevated temperature (+10 °C), and supplied with ^{13}C and ^{15}N isotopes to test for treatment effects on the relative uptake of ammonium, alanine or tri-alanine.
- Both grass species took up relatively more N derived from ammonium than from alanine or tri-alanine when grown under ambient conditions in monoculture. In contrast, when grown in mixtures, *A. odoratum* took up N derived from the three N forms in equal amounts, whereas *F. ovina* switched to tri-alanine as an alternative N form. Under warmed conditions, both species took up the N forms equally, irrespective of competition treatments.
- We have shown that grass species grown in mixture and under ambient conditions reduce competition by acquiring different N forms. Warming increased the availability of inorganic N in the soil and therefore deregulated the need for differential uptake of N forms.

Keywords:

amino acid, peptide, nutrient, coexistence, niche differentiation

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47 **Introduction**

48 Soil nitrogen (N) availability is one of the most important growth-limiting factors in natural or semi-
49 natural grasslands (Vitousek and Howarth 1991). There is growing evidence that increasing
50 temperatures due to global warming will accelerate rates of soil N turnover in these and other
51 temperature-limited ecosystems (Bai et al. 2013; IPCC 2013; Prescott 2010; Zhang et al. 2008), leading
52 to increased soil N availability and a shift in the dominant N form from dissolved organic N (DON) to
53 soluble inorganic N (DIN) (Bai et al. 2013; Rennenberg et al. 2009; Saxe et al. 2001). In addition to
54 changing climate, changes in grassland land use, such as shifts in management intensity or grazing
55 density, also modify microbial communities and rates of soil N turnover, causing shifts in the availability
56 of different N forms (de Vries et al. 2012; Medina-Roldan et al. 2012), with the amount of DON relative
57 to DIN being greater in low than in high productivity, intensively managed grasslands (Bardgett et al.
58 2003; Christou et al. 2005; Schimel and Bennett 2004).

59 It is well established that plant species are able to take up soil N in a range of forms, either as inorganic
60 N, in the form of ammonium (NH_4^+) and nitrate (NO_3^-), or as organic N, in the form of urea, amino acids
61 and peptides (Näsholm et al. 2009; Näsholm and Persson 2001; Sauheitl et al. 2009b; Soper et al. 2011).
62 Although grasses are relatively plastic with regard to their use of different N forms (Falkengren-Grerup
63 et al. 2000; Sauheitl et al. 2009b), it has been suggested that under N limiting conditions grass species
64 acquire contrasting forms of N, which appear to be linked to their growth strategies (Kahmen et al. 2006;
65 Weigelt et al. 2005). This plasticity in acquiring different N forms has been proposed to be a strategy
66 for co-existing plant species to reduce niche overlap, and therefore to avoid competition for the same
67 limiting resource (Ashton et al. 2010; McKane et al. 2002). Results from studies testing for niche
68 partitioning based on chemical forms of N in grasslands, however, are mixed: some report differences
69 in N forms taken up by co-existing grassland plant species (Ashton et al. 2010; Kahmen et al. 2006),
70 whereas others do not (Ashton et al. 2008; Harrison et al. 2007).

71 Given that climatic conditions are known to regulate the availability of different N forms, it is likely that
72 modified N availability due to warming will also lead to a shift in N forms taken up by plants. Indeed,
73 Warren (2009) reported that *Eucalyptus pauciflora* Sieber ex Spreng. took up more glycine than nitrate

at low temperatures, whereas the opposite was true when temperatures were higher due to changed N pool turnover rates. Similarly, in arctic tundra, glycine uptake by herbs was reduced by long-term warming (Sorensen et al. 2008), whereas glycine acquisition by the grass *Deschampsia flexuosa* (L.) was found to increase with warming (Andresen et al. 2009). Given this, our goal was to test how warming impacts the uptake of different N forms by grass species with contrasting life history strategies, and whether this influences their competitive interactions. We focused on two grass species that co-exist in low productivity, semi-natural temperate grassland: the slower-growing species *Festuca ovina* L. and the faster-growing species *Anthoxanthum odoratum* L. (Elberse and Berendse 1993; Ryser and Wahl 2001; Schippers and Olff 2000; Schippers et al. 1999). These species have previously been shown to differ in their acquisition of organic and inorganic N forms in monoculture. *Festuca rubra* L., as a close relative to *F. ovina*, displays a selective placement in nutrient-rich patches with shorter roots and has been reported to take up relatively more inorganic than organic N, whereas *A. odoratum*, with its longer roots spread more evenly in the soil, relies equally on both forms (Elberse and Berendse 1993; Harrison et al. 2007; 2008; Mommer et al. 2011; Schippers and Olff 2000; Weigelt et al. 2005).

We hypothesised that: (i) when grown in monoculture, the two grass species would preferentially take up N derived from different N forms reflecting their differing life history strategies; (ii) when grown in mixture, this difference is amplified to avoid competition for soil N; and (iii) at warmer temperatures preferences for N derived from different forms becomes less important for *F. ovina* and *A. odoratum* due to increased availability of DIN compared to ambient temperatures. To test these hypotheses, we conducted a factorial pot experiment, in which *F. ovina* and *A. odoratum* were grown either in monocultures or mixtures at both ambient or elevated temperature, and were supplied with ^{13}C and ^{15}N labelled compounds to test the relative uptake of ammonium (as a representative form of inorganic N), alanine (amino acid) or tri-alanine (peptide).

Materials and Methods

Experimental Setup

We established a pot experiment using field soil collected from a grassland site at Abergwyngregyn, Gwynedd, North Wales, UK (53° 13' 27" N, 4° 00' 50" W, 320 m a.s.l.), as described by Farrell et al. (2011a). Briefly, the selected site is classified as a semi-natural *Agrostis-Festuca* grassland, based on the UK National Vegetation Classification (Rodwell 1992), and is dominated by the grasses *Agrostis canina* L., *Agrostis capillaris* L., *A. odoratum* and *F. ovina*, and the herbs *Potentilla erecta* (L.) Raeusch. and *Galium saxatile* L.. The soil is an organic matter rich Cambic Podzol with an acidic pH (4.8) and is representative of a typical semi-natural, sheep-grazed upland grassland in the western United Kingdom (Bardgett et al. 2001). The dissolved N pool is rich in organic N (301 ± 74 mg m^{-2}), whereas concentrations of NH_4^+-N (73.4 ± 36.8 mg m^{-2}) and NO_3^--N (0.6 ± 0.5 mg m^{-2}) are lower (data refer to a depth of 15 cm, published in Wilkinson et al. (2015)). The climate, measured at sea level at a distance of ca. 1 km from the sampling site, is cool and wet with a mean annual air temperature of 10.7 °C, soil temperature of 11 °C (at 10 cm depth) and rainfall of 1250 mm. In spring 2013, soil from the field site was excavated from the rooting zone down to 15 cm depth. Soil was transported back to the laboratory where stones and roots were removed. After passing through a 4 mm sieve, the soil was thoroughly mixed and stored afterwards at 4 °C until the start of the experiment.

We selected two grass species: *A. odoratum* and *F. ovina*. Both species co-exist at the site, although *A. odoratum* is generally more abundant in more productive grasslands, and *F. ovina* is more abundant in lower productivity grasslands (Grime et al. 2007). In April 2013, seeds (Emorsgate Seeds, King's Lynn, UK) of *A. odoratum* and *F. ovina* were germinated in a 1:1 mixture (*v*:*v*) of a low fertility compost (No 1; John Innes Manufacturers Association, Reading, UK) and horticulture sand (Keith Singleton Horticulture, Egremont, UK) at ambient temperatures in a greenhouse at The University of Manchester. Due to differences in germination and establishment rates, *A. odoratum* was sown two weeks later than *F. ovina* in order to produce uniformly sized seedlings. Trays were watered every second day with tap water without using any additional fertiliser. After 32 (*A. odoratum*) and 46 (*F. ovina*) days, seedlings with an average height of 9 cm were allocated to 3 intra- and interspecific planting treatments, each with two individual plants: i) *F. ovina* monoculture; ii) *A. odoratum* monoculture; and iii) *F. ovina* and *A. odoratum* mixture. Care was taken to ensure that the height of individuals in each of the 192 pots (side length = 9 cm, used height = 7 cm, average soil volume = 0.567 l) was similar. Immediately after

planting, pots of each treatment were randomly assigned to two temperatures in controlled growth cabinets (day length 16 h), namely: 12 °C, representing ambient growing season temperature, and 22 °C, representing warming. The ambient temperature refers to an average temperature during growing seasons at the field site (13.7 °C at sea level, implying approximately 12 °C at the field site). Warming of 10 °C was used as an approach to extrapolate the climate sensitivity of N availability and uptake in a model ecosystem. Pots were randomly relocated within cabinets twice per week.

Pots were irrigated with tap water bi-weekly (ambient: 50 ml pot^{-1} $week^{-1}$; warming: 100 ml pot^{-1} $week^{-1}$, total dissolved N in tap water < 0.4 mg l^{-1}), with differences in irrigation between the two treatments accounting for estimated greater evapotranspiration due to increased temperature and plant biomass in the warmed compared to ambient treatment. The difference in N input through irrigation between the treatments due to the different amount of water (ambient: < 0.16 mg pot^{-1}; warming: < 0.32 mg pot^{-1}) was negligible compared to total N per pot (approximately 2 g N pot^{-1}). The height of each seedling (longest shoot) was measured weekly.

Isotope labelling and harvest of plant biomass

Labelling of soils to measure uptake of different N forms was performed after 71 days, at a period when shoot height had remained stable for several weeks. Twelve replicate pots of each planting × temperature treatment were randomly allocated to the following three labelling treatments (72 out of 192 pots): i) $^{15}NH_4Cl$ (98% ^{15}N, Cambridge Isotope Laboratories, Andover, MA, USA); ii) alanine (97-99% U-^{13}C, 97-99% ^{15}N, Cambridge Isotope Laboratories); and iii) tri-alanine, (97-99% U-^{13}C, 97-99% ^{15}N, CK Gas Products, Ibstock, UK). Nitrate concentration in the original field soil was negligible compared to DON and ammonium (Wilkinson et al. 2015), and therefore, nitrate was not used for labelling. There were 4 replicates for each treatment-labelling combination. The other 120 pots were treated with an unlabelled N solution (18 µmol N pot^{-1}), from which 8 pots were analysed for natural abundance assessments. Each labelling solution (18 µmol N pot^{-1}) was made up of equal concentrations (6 µmol N pot^{-1} for each N form) of ammonium, alanine and tri-alanine, in which one of the three N forms was isotopically labelled. This enabled us to test for preferential uptake by individual plant species and soil microbes (Harrison et al. 2007; Weigelt et al. 2005). The use of dual-labelled $^{13}C^{15}N$ compounds is

generally, but not unequivocally, considered to be a good indication whether amino acids and peptides such as alanine and tri-alanine are taken up by plants directly as organic N, or as inorganic N after microbial mineralisation, as confirmed by enrichment of plant tissue with both ^{13}C and ^{15}N (Näsholm et al. 1998). The amount of N added to each pot was considered to be sufficient to allow for detection of ^{13}C and ^{15}N within plant and microbial biomass, but keeping the possible N fertilisation effect on plant growth to a minimum (18 µmol N pot^{-1} (0.3 kg N ha^{-1}) < N_{H20} = 490 µmol N pot^{-1}). Within each pot, the labelling solution (20 ml) was injected at 5 different locations, equally distributed over the soil depth, using a glass syringe (S Murray & Co, Surrey, UK). Pots were randomly labelled over a period of four days.

Three hours after labelling, pots were destructively harvested and plants were separated from the soil. A chase period of 3 hours was chosen to reduce plant uptake of recycled mineralised organic N, but to provide sufficient time to detect $^{13}C^{15}N$ in roots and shoots (Warren 2012). Roots were first washed with deionised water and then rinsed with 0.5 M $CaCl_2$ to remove ^{13}C and ^{15}N in the apoplast and sorbed to the cell wall. Roots of the two species in the mixed treatment were distinguished from each other by their colour. Root, shoot and soil samples were dried at 65 °C for two days prior to grinding (MM 400, Retsch, Haan, Germany). Root and shoot samples of the two individuals grown in monocultures were pooled prior to grinding, whereas for mixed treatments both individuals were analysed separately. In order to determine $^{13}C^{15}N$ uptake by the soil microbial biomass, 0.5 M K_2SO_4 extractions were carried out on fumigated (amylene-stabilised $CHCl_3$, Fisher Scientific, Waltham, MA, USA) and non-fumigated soil (Brookes et al. 1985). The extracts were freeze-dried prior to further processing (ScanVac CoolSafe 55-4 Pro, Lynge, Denmark). Microbial $^{13}C^{15}N$ uptake was calculated as the respective differences between fumigated and non-fumigated samples. The differences were divided by the corrections factors k_{EN} = 0.50 and k_{EC} = 0.35 to estimate microbial biomass C and N values (Carter 2008). However, we acknowledge the uncertainty in the values when used for isotopic labelling experiments (Glanville et al., 2016). For unknown reasons, we did not detect any uptake of ^{13}C or ^{15}N by the microbial biomass under warming, so data are not presented for this treatment.

Root, shoot, soil and microbial extract samples were analysed for $^{12/13}C$ and $^{14/15}N$ concentrations at the NERC Life Sciences Mass Spectrometer Facility, Centre for Ecology and Hydrology, Lancaster, UK, (precision for working standards better than 0.46 ‰ (^{13}C) and 6.92 ‰ (^{15}N)). Samples were combusted in a Carlo Erba NA1500 elemental analyser (Thermo Scientific, Waltham, MA, USA). The resultant CO_2/N_2 from combustion and reduction was analysed for $\delta^{13}C/^{15}N$ using an isotope ratio mass spectrometer (IRMS; Dennis Leigh Technologies, Sandbach, UK). $^{13}C^{15}N$ excess values were calculated by using formulas (1) and (2).

$$R_{sample} = [(\delta^{13}C/1000) + 1] * R_{PDB} \quad (1)$$

where R is the ratio of ^{13}C/of ^{15}N to ^{12}C/to ^{14}N and R_{PDB} is the natural abundance standard for C and N.

$$\text{Atom\%} = (R/R+1) * 100 \quad (2)$$

Atom % excess values were calculated by subtracting control atom % values from treatment atom % values. Natural abundance levels of ^{13}C in our samples were highly variable. We therefore used the lowest natural abundance atom % value to calculate ^{13}C excess values.

Soil nutrients, microbial biomass and root length

Immediately after plants were harvested, fresh soil samples were extracted with deionised water (1:7.1 *w/v* soil:extractant; extraction time = 10 min) to measure total dissolved N and inorganic N as either nitrate (NO_3^-) or ammonium (NH_4^+). Extracts were measured with an AutoAnalyzer 3 (SEAL Analytical, Fareham, UK). DON was calculated after subtracting water-soluble inorganic N from total water-soluble N. Dissolved organic carbon (DOC) was measured in water extracts using a TOC-L analyser (Shimadzu, Kyoto, Japan). For determining microbial C (C_{mic}) and N (N_{mic}), chloroform-fumigated (fumigation time = 24 h) and non-fumigated soil samples were extracted with 0.5 M K_2SO_4 (1:2.5 *w/v* soil:extractant; extraction time = 60 min) (Brookes et al. 1985), and total soluble organic carbon and N in K_2SO_4-extracts were measured with a TOC-L (Shimadzu, Kyoto, Japan) and an AutoAnalyzer 3 (SEAL Analytical, Fareham, UK), respectively. In labelled soil samples, pH was measured in 0.01 M $CaCl_2$ (FE20, Mettler-Toledo, Schwerzenbach, Switzerland). Root samples from pots that were not used for the labelling experiment were analysed for their diameter and length using

an Epson Expression 11000 XL, scanner (Nagano, Japan) and WinRHIZO Pro 2013a (Regent Instruments Inc., Quebec, CA).

Statistical analysis

Data were analysed after log-transformation by ANOVA using a linear model (significant at $P < 0.05$) in R 3.02 (R Development Core Team, Vienna, AT). The initial shoot height (analysis of root and shoot biomass) and final biomass ($^{12/13}C^{14/15}N$ values) were included in the models to account for differences between pots. Selected differences between treatments and soils were pair-wise tested using contrasts based on *t*-tests (significant at $P < 0.05$).

Results

Soil N availability and microbial biomass

Concentrations of inorganic N (NH_4^+ and NO_3^-) were influenced by planting and warming treatments (Table 1). Concentrations of NH_4^+ ($F_{(2,181)} = 36.6$, $P < 0.001$) and NO_3^- ($F_{(2,181)} = 44.9$, $P < 0.001$) were lowest in soil planted with *A. odoratum*, followed by mixtures and *F. ovina* monocultures. Soil concentrations of NH_4^+ ($F_{(1,181)} = 88.0$, $P < 0.001$) and NO_3^- ($F_{(1,181)} = 59.9$, $P < 0.001$) were greater in the warming than in the ambient treatment. Dissolved organic N (DON) was lower in the ambient treatment than the warming treatment ($F_{(1,181)} = 5.2$, $P = 0.023$). Pair-wise comparisons for DON were, however, only significant in *A. odoratum* monocultures ($P = 0.007$) and not in the other planting treatments (*F. ovina* monocultures: $P = 0.171$; mixtures: $P = 0.895$). Dissolved organic carbon (DOC) was changed by the planting treatment ($F_{(2,186)} = 4.7$, $P = 0.010$): DOC concentrations were, or tended to be greater in *A. odoratum* than in *F. ovina* monocultures (ambient: $P = 0.054$; warming: $P = 0.028$). There was, however, no warming effect on soil DOC, and neither total soil carbon (C_{tot}), nitrogen (N_{tot}) or pH were affected by the warming and planting treatments (Table 1). Soil water concentration at the end of the experiment was greatest in warmed *F. ovina* monoculture, whereas no differences between the other treatments were observed.

Both microbial biomass C ($F_{(1,167)}$ = 26.6, P < 0.001) and N ($F_{(1,167)}$ = 4.1, P = 0.045) were greater in the ambient than in the warming treatment (Table 1). However, effects of warming on microbial biomass C (temperature × planting: $F_{(2,167)}$ = 11.8, P < 0.001) and N (temperature × planting: $F_{(1,167)}$ = 5.5, P = 0.005) varied with planting design: under ambient conditions, microbial biomass C was lowest in mixtures, whereas under warmed conditions it was smallest in *F. ovina* monocultures. Similarly, microbial biomass N under warming was lower in *F. ovina* monocultures than in mixtures, but not different from *A. odoratum* monocultures. We observed no differences in microbial N between the planting treatments under ambient conditions.

Root and shoot biomass

Elevated temperature on average doubled the shoot biomass of *A. odoratum*, whereas warming only marginally influenced shoot biomass of *F. ovina* (temperature × species: $F_{(1,247)}$ = 91.9, P < 0.001, Fig. 1a). The planting treatment only affected shoot biomass of *A. odoratum* in the warming treatment (temperature × planting: $F_{(1,247)}$ = 4.5, P = 0.035): *A. odoratum* shoot biomass per plant was 50% higher in mixtures than in monocultures (competition ratio (CR) = 1.5 ± 0.1). However, total shoot biomass per pot (2 plants) in warmed *A. odoratum* monocultures did not differ from the total biomass of the two species in mixtures (P = 0.745). Planting treatment had no effect on the shoot biomass of *A. odoratum* under ambient temperature (CR = 0.9 ± 0.1), or on shoot biomass of *F. ovina* under ambient (CR = 1.0 ± 0.1) or warmed conditions (CR = 1.2 ± 0.1).

Warming decreased root biomass of *F. ovina* in monoculture and mixtures, but it had no effect on root biomass of *A. odoratum* in monoculture, although it increased root biomass of this species in mixtures (temperature × species: $F_{(1,247)}$ = 49.7, P < 0.001, Fig. 1a). As a result, under elevated temperatures, root biomass of *A. odoratum* was more than four times greater than of *F. ovina*, whereas root biomass did not differ between the two species under ambient conditions (Fig. 1a). Warming decreased the root:shoot ratio of the test species ($F_{(1,247)}$ = 178.9, P < 0.001): the effect of temperature on the root:shoot ratio of *F. ovina* was greater than on that of *A. odoratum* (temperature × species: $F_{(1,247)}$ = 39.8, P < 0.001), leading to a significantly higher root:shoot ratio of *A. odoratum* than of *F. ovina* in the warming treatment. There was no significant planting effect on root biomass or root:shoot ratio of either species

($F_{(1,247)}$ = 1.6, P = 0.201, $F_{(1,247)}$ = 0.8, P = 0.383). As with root biomass, root length of *F. ovina* was least in the warming than in the ambient treatment, whereas root length of *A. odoratum* grown in mixtures was greater under warming than ambient conditions (temperature × planting: $F_{(1,143)}$ = 55.4, P < 0.001, Fig. S1A). No warming effect on root length was observed in *A. odoratum* monocultures; hence, root length of *A. odoratum* was greater than of *F. ovina*, but only under warmed conditions.

N concentrations in root and shoot

Temperature effects on shoot N differed between the two grass species (temperature × species: $F_{(1,87)}$ = 37.1, P < 0.001): shoot N in *A. odoratum* was greater under warming than under ambient temperature, whereas for *F. ovina* no effect of warming was detected (Fig. 1b). Planting design also influenced the two species differently (planting x species: $F_{(1,87)}$ = 19.4, P < 0.001). Although pair-wise comparisons were not significant, shoot N in *A. odoratum* tended to be greater in mixtures than in monocultures, whereas it was the other way around in *F. ovina*. Hence, shoot N concentrations under ambient and monoculture conditions were higher in *F. ovina* than in *A. odoratum* (P = 0.004), whereas N concentrations were lower in *F. ovina* than in *A. odoratum* in warmed mixture (P = 0.001). In general, root N concentrations were greater under elevated than under ambient temperature ($F_{(1,87)}$ = 9,1, P < 0.001, Fig. 1b). However, pair-wise comparisons revealed that this response to warming was only significant in *A. odoratum* roots grown in mixtures (P = 0.015).

$^{13}C^{15}N$ excess values in root and shoot biomass

Enrichment of plant material, measured as absolute ^{15}N excess values, differed strongly between the two grass species (roots: $F_{(1,71)}$ = 21.1, P < 0.001; shoots: $F_{(1,71)}$ = 72.9, P < 0.001). On average, ^{15}N excess values in roots and shoots of *A. odoratum* were higher than in those of *F. ovina*, which is indicative of greater uptake of all N forms (Fig. 2, Table 2). Differences in ^{13}C excess values between the two species, however, were only weakly or not significant (roots: $F_{(1,47)}$ = 3.6, P = 0.064; shoots: $F_{(1,71)}$ = 5.8, P = 0.020), although there was a trend towards higher ^{13}C concentrations in *A. odoratum* than *F. ovina* (Table 2).

Plant uptake of N was affected by chemical N form (roots: $F_{(2,71)} = 18.1$, $P < 0.001$; shoots: $F_{(2,71)} = 22.2$, $P < 0.001$), planting design (roots: $F_{(1,71)} = 10.0$, $P = 0.002$; shoots: $F_{(1,71)} = 22.0$, $P < 0.001$) and warming treatment (roots: $F_{(2,71)} = 6.5$, $P = 0.013$; shoots: $F_{(2,71)} = 57.1$, $P < 0.001$). Most interestingly, planting treatment influenced the uptake of N forms by *A. odoratum* under ambient conditions: in monoculture, ^{15}N excess rates in *A. odoratum* roots and shoots were greater for ammonium than alanine (roots; shoots: $P < 0.001$; $P < 0.001$) or tri-alanine ($P = 0.066$; $P = 0.023$), whereas in mixture uptake of N derived from tri-alanine was greater than from ammonium ($P = 0.049$; $P = 0.860$) or alanine ($P = 0.011$; $P = 0.005$). This shift in N forms taken up by *A. odoratum* can mainly be deduced from a smaller ammonium uptake in mixture than in monoculture ($P < 0.001$; $P = 0.016$), whereas we observed no difference in uptake of N derived from tri-alanine between the planting treatments. In *F. ovina* roots and shoots grown at ambient conditions, differences between N forms were less obvious than for *A. odoratum*. In monoculture, uptake of N derived from alanine was less than for ammonium ($P < 0.001$; $P < 0.001$) and tri-alanine ($P = 0.020$; $P = 0.083$). In mixture, we observed no difference in uptake of different N forms on the basis of ^{15}N excess in *F. ovina* roots, but values in shoots were greater when plants were labelled with ammonium than with alanine ($P = 0.001$) or tri-alanine ($P = 0.032$). Root uptake of ^{13}C was also affected by the chemical form ($F_{(1,47)} = 16.5$, $P < 0.001$); under ambient conditions ^{13}C excess values were higher when plants were labelled with tri-alanine than with alanine (Table 2).

Warming changed the observed planting effects on ^{15}N uptake under ambient conditions (temperature × form in roots: $F_{(2,71)} = 3.0$, $P = 0.056$; shoots: $F_{(2,71)} = 6.6$, $P = 0.002$); in general, we detected no differences in ^{15}N and ^{13}C excess values in both species between the applied N forms under warmed conditions (Table 2, Fig. 2). As an exception to this pattern, ^{15}N excess values for *F. ovina* roots in monoculture were greater for tri-alanine than ammonium ($P = 0.033$) or alanine ($P = 0.023$), and for *A. odoratum* shoots, ^{15}N excess values were greater for ammonium than alanine ($P = 0.033$).

We found significant correlations between ^{13}C and ^{15}N excess values in *A. odoratum* roots for alanine ($R^2 = 0.287$, $P = 0.027$) and tri-alanine ($R^2 = 0.401$, $P = 0.011$, Fig. 3). The slope of the alanine correlation line (m = 0.9) was slightly steeper than that of tri-alanine (m = 0.5), indicating that direct uptake of alanine was greater than for tri-alanine, that the proportion ^{13}C lost in plant respiration was greater when

acquired as tri-alanine than when acquired as alanine, or that the C and N from the compounds partitioned differently between roots and shoots. In *F. ovina* roots, we observed no correlations between ^{13}C and ^{15}N excess values (alanine: $R^2 = 0.022$, $P = 0.557$; tri-alanine: $R^2 < 0.001$, $P = 0.946$). The slopes of the correlation lines separately calculated for each planting and warming treatment did not differ from the patterns described above.

$^{13}C^{15}N$ excess values in microbes and soil

We observed significant differences under ambient conditions in microbial ^{15}N excess values between the three N forms applied ($F_{(2,54)} = 5.1$, $P = 0.010$); ^{15}N excess values in microbes were greater when applied as tri-alanine than as ammonium (*F. ovina* monoculture: $P = 0.011$; *A. odoratum* monoculture: $P = 0.054$; mixture: $P = 0.056$, Fig. 4). In *F. ovina* monoculture only, microbial ^{15}N excess values originating from tri-alanine were also higher than those from alanine ($P = 0.042$). ^{15}N excess values in the case of ammonium solution application were close to zero, indicating that uptake of this N form by microbes was low. No data are presented for the warming treatment, as we did not detect any uptake of ^{13}C or ^{15}N by the microbial biomass under warming,

In bulk soil samples, greater ^{15}N excess values were observed when applied as tri-alanine than as ammonium or alanine ($F_{(2,54)} = 42.9$, $P < 0.001$). An exception to this pattern was that no difference in soil ^{15}N excess values between the three labelling solutions was recorded for ambient *A. odoratum* monocultures (Fig. 4). Soil ^{13}C data confirmed the pattern described above; when applied as tri-alanine, soil excess values were greater, or equal, than when applied as alanine ($F_{(1,34)} = 29.8$, $P < 0.001$, Table 2).

Discussion

The aim of this study was to test for the effects of warming on the uptake of different N forms and competitive interactions of two grass species of temperate grasslands with contrasting functional traits. Our first hypothesis was that the two grass species take up N derived from different N forms when grown in monocultures, and this difference is greater in mixture to avoid competition for soil N. In contrast to this hypothesis, and to previous studies on inorganic and organic N uptake (Harrison et al.

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2007; 2008; Weigelt et al. 2005), we found that both *F. ovina* and *A. odoratum* took up more N derived from ammonium from alanine when grown in monocultures. When grown in mixture, however, *A. odoratum*, but not *F. ovina*, switched from taking up more N from ammonium than alanine, to greater uptake of N derived from tri-alanine than from alanine or ammonium. The difference in N uptake between these species in mixture is reflected in their functional root traits: *Festuca* is known to place roots selectively in nutrient-rich hotspots, whereas *Anthoxanthum* spreads its roots more evenly in soil, allowing uptake of a greater variety of N forms (Mommer et al. 2011). This suggests that when grown in mixture, *F. ovina* was more competitive than *A. odoratum* in taking up the same N form as in monoculture, thereby reducing *A. odoratum's* ammonium uptake. Competition for N between plants and microbes was presumably strong in both monocultures and mixtures, as indicated by higher ^{15}N excess values in microbes compared to plants, and, therefore, *A. odoratum* could not compensate the reduced ammonium uptake by acquiring more N derived from alanine or tri-alanine. Moreover, we exclude that competition between microbes and plants explains the decreased ammonium uptake by *A. odoratum* as this would likewise have affected ammonium uptake by *F. ovina*. Similarly, ammonium immobilisation by microbes did not differ between planting treatments under ambient conditions, as evidenced by the lack of change in microbial ^{15}N excess values derived from added ammonium. Our data, therefore, suggest that this shift in N uptake by *A. odoratum* was mainly induced by a lower competitiveness for ammonium in comparison with *F. ovina*, which lends support to the idea that acquisition of different N forms contributes to coexistence of competing grass species (Ashton et al. 2010; Kahmen et al. 2006; McKane et al. 2002).

As hypothesised, we found that warming changed N use by the two plant species, in that we detected no difference in uptake of the different N forms when they were grown in mixture compared to monocultures in this treatment. It is possible that increased soil inorganic N availability under warming compensated for the need for niche differentiation on the basis of N form, which was detected in mixtures under ambient conditions. Indeed, nitrate and ammonium concentrations were greater in the warming than ambient treatment, which is likely to be due to accelerated organic matter turnover in this organic-rich grassland soil (Bai et al. 2013; Prescott 2010; Rennenberg et al. 2009; Zhang et al. 2008). An alternative mechanism is that warming influenced the competitiveness of the two grass species,

364 which might have weakened in the requirement for niche differentiation; whereas under ambient
365 conditions the biomass of the two species was similar, *A. odoratum* clearly outcompeted *F. ovina* in the
366 warming treatment. In a study conducted by Schippers and Olff (2000), *A. odoratum* was still more
367 vigorous than *F. ovina* at 15 °C, indicating that the optimum temperature of *F. ovina* is rather closer to
368 12 °C than to 22 °C. The lower root:shoot ratio and plant N concentrations of *F. ovina* compared to *A.*
369 *odoratum* indicate that the differences in competiveness between our test species can be related to a
370 more effective nutrient uptake by *A. odoratum* compared to *F. ovina* in the warmed treatment (Mommer
371 et al. 2011). Otherwise, the low root biomass of *F. ovina* under warming might have been a consequence
372 of the high soil water availability in the *F. ovina* monoculture relative to *A. odoratum* and mixtures. This
373 would mean that due to sufficient water availability in the topsoil there was no need for *F. ovina* to
374 allocate resources to root growth and hence *F. ovina* was likely less competitive in taking up nutrients
375 compared to *A. odoratum*. With its higher root density, *A. odoratum* is likely to be also more competitive
376 under water-limiting conditions, as predicted to increase in frequency with climate change (IPCC 2013);
377 this question, however, was not tested in our experiment and needs further investigation. It is possible
378 that, in the long term, niche partitioning on the basis of uptake of different forms of N will occur in the
379 real world under warming, especially due to acclimatisation of microbial activity and increased plant
380 biomass production (Lu et al. 2013; Luo et al. 2001) or immigration of other species (Klanderud and
381 Birks 2003; Parolo and Rossi 2008). We therefore conclude, in accordance with our third hypothesis,
382 that warming reduces the need for niche differentiation on the basis of N form in grass species, at least
383 in the short timescale of our study.

384 Even though our data show how competition and temperature influence the uptake of N forms by *F.*
385 *ovina* and *A. odoratum*, the applied $^{13}C^{15}N$ labelling technique has some limitations. First, it is possible
386 that N forms other than those we supplied to soil might have also been important for plant nutrition.
387 Unlike in the field (Wilkinson et al. 2015), concentrations of nitrate were higher than ammonium or
388 DON in soil of the present experiment. We found that soil nitrate concentrations were reduced under
389 ambient conditions by the presence of *A. odoratum*, indicating that nitrate was a significant part of
390 nutrition for *A. odoratum*. Soil concentrations of nitrate in mixtures, however, suggest that *A. odoratum*
391 did not increase its nitrate acquisition when grown alongside with *F. ovina*. Hence, even though *A.*

392 *odoratum* may have taken up a significant amount of nitrate, our conclusions, gained from the reduced
393 ammonium uptake in mixture compared to monoculture, would not be different. Second, correlations
394 between ^{13}C and ^{15}N excess values in *A. odoratum* roots and $^{13}C^{15}N$ excess values in microbes may
395 indicate that a higher fraction of tri-alanine, compared to alanine, was first mineralised then taken up as
396 inorganic N, as similarly reported by Farrell et al. (2013). We presume, however, that direct uptake of
397 tri-alanine was nevertheless an important source for plant nutrition: on the one hand we found higher
398 plant ^{13}C excess values for tri-alanine than for alanine, indicating that direct uptake of the peptide was,
399 in absolute numbers, higher than of the monomer; on the other hand, differences between ^{13}C and ^{15}N
400 correlations might be explained by faster within-plant mineralisation of tri-alanine compared to alanine
401 (Hill et al. 2011; Warren 2012). In other words, residual carbon, including ^{13}C, might have been respired
402 to a higher extent when applied as peptide than as amino acid, resulting in a higher $^{15}N^{13}C$ ratio. To
403 reduce such uncertainties about direct uptake of labelled isotopes in future experiments, the application
404 of other techniques might be helpful, such as compound-specific stable isotope measurements (Sauheitl
405 et al. 2009a), position-specific labeling (Apostel et al. 2013) and the use of ^{14}C-labelled isotopes (Hill
406 et al. 2013). However, all available techniques are subject to some caveats and assumptions. Third, pool
407 dilution of applied labelling solutions has to be taken into account when interpreting ^{13}C and ^{15}N uptake
408 in plant samples (Jones et al. 2005). At field conditions, concentrations of alanine, tri-alanine and other
409 amino acids and peptides competing for the same root transporters were smaller than those of
410 ammonium in the soil used during the present pot experiment (Farrell et al. 2011a; Farrell et al. 2011b).
411 Hence, the chance of a plant root to take up labelled ammonium was smaller in comparison with labelled
412 N derived from organic forms. Otherwise, considering the faster turnover rates of amino acids and
413 peptides comparing to ammonium, plants have much more capacity to take up $^{15}N\text{-}NH_4^+$ during the
414 labelling period. Taking pool dilution into account by multiplying soil N pools reported by Farrell et al.
415 (2011b) by ^{15}N excess values recorded within root or shoot tissue, we estimate that 30-100x more ^{15}N-
416 NH_4 was recovered in plant material than the tested organic ^{15}N forms. Likely, differences in N uptake
417 in ambient monocultures would be even more distinct when considering pool dilution, whereas shifts in
418 N uptake by *A. odoratum* grown in mixture would be less obvious. However, as this correction would

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likewise apply for both monocultures and mixture, the relative difference in ammonium uptake would not be different and hence, our main conclusions from this experiment are still likely to be true.

Conclusions

Our data show that grass species grown in mixture and under ambient conditions reduce competition by taking up different N forms. Thereby, N derived from organic forms as amino acids and peptides can play a major role for plant nutrition. Hence, the possibilities for a plant species to create its own niche are manifold and may include intricacies such as acquiring different N forms. Increased availability of inorganic N due to warming deregulated the need for differential uptake of N forms. Hence, we conclude that uptake of different N forms is mainly important at nutrient-limiting conditions. Besides taking up different N forms, grass species have also been shown to coexist through spatiotemporal shifts in nutrient acquisition (McKane et al. 1990; Pornon et al. 2007). Whereas we exclude spatiotemporal shifts in N uptake as a source for niche differentiation in the present study, these other strategies might explain why in some field studies niche differentiation by taking up different N forms has been reported (Ashton et al. 2010; Kahmen et al. 2006), whereas in others it has not (Ashton et al. 2008; Harrison et al. 2007).

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Figure legends

Fig. 1 a: Average root and shoot biomass per individual (g, ± SE, n = 32), separately for each temperature and planting treatment. Total root length is shown in Fig. S1. b: Average nitrogen (N) concentrations in root and shoot biomass (%, ± SE, n = 12). Please note that the y-axis (shoot N) starts at 2%. a & b: Values of the two individuals in the monoculture treatment were pooled prior analysis. Significant (P < 0.05) pair-wise comparisons are indicated by *: difference between monoculture and mixed treatments within the same species and temperature treatment; s: difference between species within the same temperature and competition treatment; w: difference between temperature treatments within the same species and competition treatment.

Fig. 2 Average ^{15}N excess rates in root (a) and shoot biomass (b) after a chasing period of 3 hours (µmol g^{-1}, ± SE, n = 4), separately shown for NH_4^+, alanine and tri-alanine. Differences between the applied tracer solutions within a given treatment combination (column) are indicated by different lower/upper case letters (all P < 0.05). Average ^{13}C excess values are shown in Table 2. Please note the different scales between the two species.

Fig. 3 Relationship between ^{13}C and ^{15}N excess values in roots of *A. odoratum and F. ovina* , separately shown for alanine (open circles) and tri-alanine (closed circles). Broken (alanine: R^2 = 0.287, P = 0.027) and solid lines (tri-alanine: R^2 = 0.401, P = 0.011) show significant regressions between the excess of both isotopes in *A. odoratum* roots. The regressions in *F. ovina* roots were not significant (alanine: R^2 = 0.022, P = 0.557; tri-alanine: R^2 < 0.001, P = 0.946). The dotted lines show the molar ^{13}C:^{15}N ratios for the nitrogen sources injected (3:1).

Fig. 4 Average ^{15}N excess rates in bulk soil and microbes after a chasing period of 3 hours (nmol g^{-1}, ± SE, n = 4), separately shown for NH_4^+, alanine and tri-alanine. Differences between the applied tracer solutions within a given treatment combination are indicated by different letters (all P < 0.05). No excess values are available for microbial samples in the warming treatment. Average ^{13}C excess values are shown in Table 2.

Supplementary Figures

Fig. S1 a: Average total root length per individual (m, ± SE, ambient *F. ovina* & *A. odoratum* monocultures: $n = 16$, all other: $n = 20$) and b: N content in roots and shoot (g, ± SE, $n = 12$), separately for each temperature and planting treatment. Significant ($P < 0.05$) pair-wise comparisons are indicated by *: difference between monoculture and mixed treatments within the same species and temperature treatment; s: difference between species within the same temperature and competition treatment; w: difference between temperature treatments within the same species and competition treatment.

Tables

Table 1 Soil properties at the end of the experiment. Values are mean ± SE. Different letters indicate significant differences between competition treatments within the same warming treatment. SWC: soil water concentration (%, n = 32, residual df = 183), C_{tot}: total carbon (mg g^{-1}, 8, 42), DOC: dissolved organic C (µg g^{-1}, 32, 186), N_{tot}: total nitrogen (mg g^{-1}, 12, 66), NH_4^+: ammonium (µg g^{-1}, 32, 181), NO_3^-: nitrate (µg g^{-1}, 32, 181), DON: dissolved organic nitrogen (µg g^{-1}, 32, 181), Mic_C: microbial carbon (mg g^{-1}, 32, 167), Mic_N: microbial nitrogen (mg g^{-1}, 32, 167). An asterisk * indicates a significant difference between warming treatments within the same competition treatment (all $P < 0.05$). Statistical analyses (F-values): Effects of temperature (T, df = 1), planting (P, df = 2) and their interactions (T × P, df = 2), levels of significances (***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$, (*): $P < 0.1$)).

	Ambient			Warming			*F*-values		
	F. ovina monoculture	*A. odoratum* monoculture	mixture	*F. ovina* monoculture	*A. odoratum* monoculture	mixture	T	P	T × P
SWC	* 41.1 ± 1.0^a	39.2 ± 1.2^a	38.0 ± 1.0^a	* 45.0 ± 1.6^a	31.2 ± 1.7^b	34.0 ± 2.1^b	9.6**	17.6***	8.3***
C_{tot}	90.1 ± 1.6^a	91.8 ± 0.7^a	92.5 ± 0.8^a	91.4 ± 0.6^a	91.2 ± 1.0^a	92.2 ± 1.9^a	<0.1	0.9	0.4
DOC	62.1 ± 5.7^a	73.3 ± 4.8^a	63.4 ± 5.1^a	56.4 ± 4.8^a	65.3 ± 2.9^b	59.2 ± 4.3^{ab}	1.5	4.7 *	0.1
N_{tot}	7.8 ± 0.1^a	7.8 ± 0.0^a	7.9 ± 0.0^a	7.9 ± 0.1^a	7.8 ± 0.1^a	7.9 ± 0.1^a	<0.1	0.8	0.5
NH_4^+	*2.7 ± 0.7^a	*1.3 ± 0.1^b	*1.6 ± 0.2^a	* 10.9 ± 1.7^a	*2.3 ± 0.3^b	*3.5 ± 0.5^b	88.0***	36.6***	8.7***
NO_3^-	*12.0 ± 2.0^a	*2.5 ± 0.5^b	*7.2 ± 1.6^a	* 57.9 ± 4.4^a	*6.5 ± 1.2^b	*14.9 ± 2.9^c	59.9***	44.9***	7.7***
DON	3.9 ± 0.3^a	*5.5 ± 1.0^a	4.0 ± 0.3^a	2.9 ± 0.6^a	*3.5 ± 0.3^a	4.1 ± 0.2^a	5.2*	2.2	2.1
Mic_C	*1.9 ± 0.1^a	*1.9 ± 0.1^a	1.6 ± 0.1^b	*1.2 ± 0.1^a	*1.4 ± 0.1^b	1.7 ± 0.1^b	26.6***	2.1	11.8***
Mic_N	*0.29 ± 0.01^a	*0.30 ± 0.02^a	0.28 ± 0.01^a	*0.23 ± 0.01^a	*0.25 ± 0.01^{ab}	0.26 ± 0.01^b	18.5***	0.7	2.9$^{(*)}$

Table 2 Mean ^{13}C excess values (nmol ^{13}C excess g^{-1}) in roots, shoots, soil and microbes, separately shown for the ^{13}C labelling solutions alanine and tri-alanine. Values are mean ± SE, n = 4. Different letters indicate significant differences between species and competition treatments within the same warming treatment and N form (all $P < 0.05$). An asterisk * indicates a significant difference between N forms within a given treatment. No excess values are available for microbial samples in the warming treatment.

	Ambient				Warming			
	F. ovina monoculture	*A. odoratum* monoculture	*F. ovina* mixture	*A. odoratum* mixture	*F. ovina* monoculture	*A. odoratum* monoculture	*F. ovina* mixture	*A. odoratum* mixture
Roots								
Alanine	279 ± 49[a]	*235 ± 60[a]	*129 ± 39[b]	259 ± 50[a]	439 ± 49[a]	558 ± 113[a]	348 ± 91[a]	440 ± 109[a]
Tri-alanine	474 ± 35[a]	*475 ± 23[a]	*268 ± 64[a]	487 ± 67[a]	567 ± 46[a]	702 ± 83[a]	397 ± 71[a]	585 ± 103[a]
Shoots								
Alanine	190 ± 82[a]	269 ± 47[a]	55 ± 111[a]	310 ± 168[a]	298 ± 41[a]	428 ± 74[a]	221 ± 149[a]	324 ± 78[a]
Tri-alanine	168 ± 50[a]	292 ± 75[a]	189 ± 134[a]	212 ± 59[a]	347 ± 46[a]	475 ± 63[a]	235 ± 58[a]	347 ± 84[a]
Soil								
Alanine	*17 ± 1[a]	11 ± 3[b]	*17 ± 3[a]		*14 ± 1[a]	*13 ± 2[a]	18 ± 2[a]	
Tri-alanine	*32 ± 2[a]	13 ± 2[b]	*27 ± 1[a]		*26 ± 2[a]	*22 ± 2[a]	25 ± 6[a]	
Microbes								
Alanine	11 ± 3[a]	-4 ± 5[a]	16 ± 9[a]					
Tri-alanine	23 ± 14[a]	17 ± 10[a]	6 ± 1[a]					