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Effect of *Lolium perenne* population differences on shoot tissue nitrogen concentrations when grown on a peat soil

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Abstract

Grass nitrogen (N) concentrations of dairy grasslands are higher on peat soil than on mineral soils. This can lead to increased N losses to air and water from dairy farming systems on peat soils. Our hypothesis was that the use of low-N perennial ryegrass (*Lolium perenne* L.) genotypes could be a means to lower grass N concentrations, when grown on peat soils. Our objective was to determine whether perennial ryegrass populations with different shoot tissue N concentrations, recorded on a sandy soil, would show different shoot tissue N concentrations and N use efficiencies (NUE) or N uptake efficiencies (NUptE) when grown on a peat soil. First, a pot experiment lasting 62 days was carried out with nine diploid and seven tetraploid populations, followed by a field experiment with two diploid and two tetraploid populations and a control lasting 30 months. Both experiments had three N fertilization levels. In the pot experiment, populations explained 3% of the variation in shoot tissue N concentration among tetraploids, 5 and 7% of the variation in NUE among diploids and tetraploids and 12% of the variation in NUptE among diploids. In the field experiment, populations explained 44% of the variation in NUptE. A higher NUE coincided with lower shoot tissue N concentrations among tetraploid populations in the pot experiment. We conclude that there is potential to alter the shoot tissue N concentrations of perennial ryegrass grown on peat soil, via selection for shoot tissue N concentrations and NUE.

Introduction

Peatlands are areas with a naturally accumulated layer of peat at the surface, and about 12% of European peatlands are drained agricultural grasslands (Byrne *et al.*, 2004; Tanneberger *et al.*, 2017). Peat is defined as 'accumulated sedentary material of which at least 30 mass percent is dead organic matter' (Tanneberger *et al.*, 2017). Grass nitrogen (N) uptake by grassland on drained peat soils is relatively high compared to mineral soils, when used in a dairy farming system. This is mainly due to a high soil N supply (SNS), caused by a higher net organic matter mineralization on drained peat soils compared to mineral soils (Vellinga and André, 1999; De Visser *et al.*, 2001). The SNS is defined as the non-fertilizer herbage shoot N uptake in the first year after cessation of N inputs, and consists therefore of organic N that is mineralized during the growing season, mineral N that is present in the soil profile in spring, N from dry and wet deposition and N fixation from free and symbiotic living microbes in the soil (Hassink, 1995). Mean SNS of dairy grassland on peat with an average lowest groundwater level of 50–80 cm below field surface has been estimated at 25.2 g m^{-2} $year^{-1}$, 24–30% higher than at mineral soils (Vellinga and André, 1999).

Vellinga and Andre (1999) observed that at the same fertilization rate, mean annual grass N concentrations were around 1.5–3.0 g kg^{-1} dry matter (DM) higher for dairy grassland on peat soils, compared to mineral soils. For dairy grassland on peat soils, grass N concentrations are often observed to exceed 24–26 g kg^{-1} DM, equal to about 150–169 g crude protein (CP) kg^{-1} DM, even under limited N fertilization regimes (Korevaar, 1986; Vellinga and André, 1999; Verloop *et al.*, 2018; Hoekstra *et al.*, 2019). At dietary CP concentrations above this range, milk and protein yields do not generally increase, while urine urea N losses increase (Kebreab *et al.*, 2002; Colmenero and Broderick, 2006; Huhtanen and Hristov, 2009). Increased urine urea N losses can in turn lead to increased ammonia losses (Smits *et al.*, 1995; Edouard *et al.*, 2019). Therefore, dietary CP concentrations above the optimal range are considered undesirable from farming and environmental perspectives.

Possible measures for optimizing dietary CP concentrations for dairy farms on peat soils are; (1) adapting N fertilization rate and timing to the seasonal SNS (Pijlman *et al.*, 2020b), (2) complementing diets with low CP components such as maize or sugar beet pulp (Smits *et al.*, 1995; Hristov *et al.*, 2015), (3) lowering the CP content of concentrates (Hoekstra

et al., 2020) or (4) using alternative crops such as plantain (*Plantago lanceolata* L.) (Pijlman *et al*., 2020a) and cattail (*Typha latifolia* L.) (Pijlman *et al*., 2019). However, there are various reasons why realizing optimal dietary N concentrations remains difficult for dairy farms on peat soils. (1) Diets are typically grass based, as the cultivation of perennial grasses is preferred over annual low-CP crops such as maize or cereals. Annual crops require more frequent tillage, which leads to undesired increased soil organic matter decomposition, nutrient losses and soil subsidence (Lohila *et al*., 2004; Taft *et al*., 2017). Grass cultivation results in a permanent coverage of the soil by vegetation, which increases the generally low load-bearing capacity for machines and cows of peat soils at high groundwater levels (Wiedow *et al*., 2016). (2) During the growing season, fertilizer inputs remain necessary to maintain or increase the nutritional value of grass and DM uptake at grazing (Peyraud and Astigarraga, 1998), which generally leads to increased grass N concentrations. Furthermore, the difference between grass N concentrations on drained peat *v*. mineral soils, at the same fertilization rate and timing, tends to increase during the growing season, since the SNS increases with growing degree days because of a greater mineralization of organic matter with increasing soil temperatures (van Eekeren *et al*., 2010; Pijlman *et al*., 2020b). As a result, controlling grass N concentrations remains difficult during the growing season on these soils. (3) Farmers increasingly manage their farms with the aim of reducing mineral losses – following environmental regulations, market demand and a transition towards a circular economy – by using relatively fewer farm inputs (Fogarassy *et al*., 2016). As a result, the use of relative low CP feeds from outside the farm, such as cereals or maize, is often restricted, which limits the possibility of complementing diets with low CP feeds.

Selection of perennial ryegrass genotypes with a high DM yield per unit of N taken up (i.e. low shoot tissue N concentration in the DM) could be a potential alternative way to optimize dietary CP concentrations and to reduce environmental impacts of dairy farming on peat soils, and could be part of a more system-based plant breeding approach (Lammerts van Bueren *et al*., 2018). According to Tas (2007), decreasing the N concentration of perennial ryegrass in grass-based diets may result in a substantial improvement in animal NUE, compared to increasing the water-soluble carbohydrates (WSC) concentration of perennial ryegrass. This is in line with the results of other studies (Moorby *et al*., 2006; Edwards *et al*., 2007), and underlines the importance of taking N concentration into account at the breeding of grasses. Different studies pointed out that there is a selection potential for N concentration in perennial ryegrass, although heritability and variation were found to be lower than that for WSC (Humphreys, 1989; Wilkins *et al*., 1997, 2000; Smith *et al*., 1998; Wilkins and Humphreys, 2003; Arojju *et al*., 2020). Over the last several decades, perennial ryegrass breeding programmes have mainly focussed on DM yield potential, persistence and digestibility (McDonagh *et al*., 2016). Selection for N uptake and concentration and N use efficiency (NUE) has received limited direct attention (Baert *et al*., 2007; Malmberg *et al*., 2023). It is furthermore known that trait expressions can be affected by management and environmental conditions (Conaghan and Casler, 2011; Parsons *et al*., 2011; Malmberg *et al*., 2023). To our knowledge, experiments on genetic variations of perennial ryegrass N concentrations at dairy grassland on peat soils have not been reported.

There are different pathways that can lead to reduced perennial ryegrass N concentrations in the harvested DM; (1) through a more efficient use of acquired N for aboveground biomass accumulation, i.e. a higher NUE or (2) through a less efficient N uptake per unit of N supply, i.e. a lower N uptake efficiency (NUptE). Here, N supply is the sum of SNS and N fertilization. Regarding the first pathway, different studies have shown that perennial ryegrass genotypes can differ in NUE (Humphreys, 1989; Wilkins *et al*., 2000; Sandaña *et al*., 2021). Regarding the second pathway, a less efficient aboveground grass N uptake could be a result of a lower total plant N uptake per unit of N supply (Wilkins and Lovatt, 1989), or a result of a shift of biomass N partitioning from aboveground to belowground biomass, at the same total plant N uptake. Brégard *et al*. (2000) observed that timothy grass populations, selected for low or high aboveground N concentrations, differed in biomass N partitioning between roots and shoots, but not in total plant N concentration.

Knowledge of pathways by which grass N concentrations are affected is of importance for the estimation of possible trade-offs within the agricultural system. For example, grasses with lower N concentrations as a result of changed N partitioning between above- and belowground biomass may result in aboveground biomass with lower leaf to stem ratios (Brégard *et al*., 2000), which will affect the nutritional value of the grass and, consequently, farm biogeochemical cycles. A higher NUE or lower NUptE as a means to reduce grass N concentration could result in increased soil N surplus, and may lead to increased soil N losses via emission of NO_3^-, N_2O or N_2 (Wilkins *et al*., 2000; Baert *et al*., 2007).

In this study, a pot experiment and field experiment were carried out consecutively with perennial ryegrass (*Lolium perenne* L.) populations differing in previously recorded N concentrations when grown on a sandy soil. In the pot experiment, 16 genetically differing populations were compared. The two diploid and two tetraploid populations which showed the largest differences in the pot experiment were compared in the field experiment over a 30-month period. Both experiments were carried out on a peat soil with three different N fertilization levels, with the objective of comparing perennial ryegrass population NUE, NUptE and N concentrations. It was hypothesized that, on a peat soil, perennial ryegrass populations selected for a different shoot tissue N concentration on a sandy soil would have a different shoot tissue N concentration and NUE or NUptE (hypothesis 1), and that populations with a high NUE (hypothesis 2) and populations with a low NUptE (hypothesis 3) would have a lower shoot tissue N concentration.

Materials and methods

Sixteen perennial ryegrass populations, consisting of nine diploid and seven tetraploid populations with either a low or a high mean shoot tissue N concentration, were selected from a large database of perennial ryegrass populations from a commercial breeding programme. This database consisted of perennial ryegrass population shoot tissue N concentrations recorded at the first three harvests in the year of seeding – 2012 or 2013 – on a sandy soil (Typic Haplohumod) (Soil Survey Staff 1999) in the Netherlands (Moerstraten, 51°32′N, 4°21′E), with a mean groundwater table greater than 120 cm below the surface. The growing season of 2012 had relatively more precipitation, while 2013 was relatively colder and had less precipitation, compared to 20-year averages (Table S1). At this site, N fertilization rates were 13, 9 and 7 g m^{-2} before the first, second and third harvest, respectively, and P and K were sufficiently applied to ensure these minerals were not limiting growth (CBGV, 2022). Nitrogen, P and

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Table 1. Mean shoot tissue N concentration recorded for the first three harvest cuts in the year of seeding on a sandy soil for the selected diploid ($n = 9$) and tetraploid ($n = 7$) populations

Diploid		Tetraploid	
Population	Shoot tissue N concentration (g kg^{-1} DM)	Population	Shoot tissue N concentration (g kg^{-1} DM)
1	19.2	10	20.8
2	20.3	11	21.3
3	20.4	12	21.9
4	20.6	13	24.5
5	20.8	14	25.1
6	21.0	15	25.4
7	21.3	16	25.6
8	25.6		
9	25.6		
Diploid mean	21.6	Tetraploid mean	23.5
Diploid S.E.M.	0.75	Tetraploid S.E.M.	0.81

S.E.M., standard error of the mean.

K were applied in the form of mineral fertilizers. Shoot tissue N concentrations of the populations differed significantly ($P = 0.004$), and mean shoot tissue N concentrations were lower for diploids than for tetraploids ($P = 0.035$) (Table 1). This database did not contain data on DM yield, NNI, NUE or NUptE of the populations.

Pot experiment

The selected nine diploid and seven tetraploid populations were tested in a pot experiment with three N fertilization levels (0, 6 or 12 g N m^{-2}) (Table 2). All treatments had three replicates, which was the required minimum for sufficient statistical power (see section 'Data analyses'). The treatments were allocated to 144 pots (size 15 × 15 × 15 cm) in a randomized complete block design. The experiment lasted 62 days. At day zero (1 April 2015), the pots were filled with a standard peat-based substrate mix in which the pH was increased to 5.8 using limestone (Jiffy, Zwijndrecht, the Netherlands) (Table 3). The pH of the substrate was increased to improve the growing conditions and mineral availability for the grass (Egan *et al.*, 2019), because peat used for substrates has typically a pH around 4.0 (Messiga *et al.*, 2022). The pots were topped with 5 mm of coarse sand. The pots were placed in a greenhouse (Moerstraten, 51°32′N, 4° 21′E) without artificial heating or light, and received water through sub-irrigation on a daily basis. Greenhouse temperature and soil moisture content were not recorded. Per pot, 38 germinating seeds were sown.

Prior to sowing, all pots received P, K and S at a rate of 35, 5 and 10 g m^{-2}, respectively, by applying a suspension of phosphoric acid (H_3PO_4), potassium hydroxide (KOH) and sulphuric acid (H_2SO_4) on top of the soil. At day 42, N was applied at a rate of 0, 6 or 12 g m^{-2} using a 50:50 mixture of liquid urea (CH_4N_2O) and ammonium nitrate (NH_4NO_3). The N fertilization level of 12 g m^{-2} was slightly higher compared to fertilization levels used for a May harvest in field conditions (about 10 g m^{-2}) (Remmelink *et al.*, 2018). A higher level was chosen because grass biomass growth and N uptake were expected to be higher in a greenhouse (Poorter *et al.*, 2016).

At days 22 and 42, aboveground biomass was harvested in order to stimulate perennial ryegrass tillering, and discarded. At day 62, grass was harvested and collected for DM (oven drying at 70°C for 48 h) and total N analyses (NEN-ISO 5983-2, Kjeldahl method, Eurofins Agro, Wageningen, the Netherlands). Grass harvests were carried out using secateurs. The stubble height after harvesting was 4 cm.

Field experiment

Two diploid and two tetraploid populations, with either the lowest or the highest mean shoot tissue N concentration in the pot experiment, were used in the field experiment. These were the diploid populations 1 and 9, and the tetraploid populations 10 and 16, renamed 2Nlow, 2Nhigh, 4Nlow and 4Nhigh, respectively, for the field experiment. A commercially available diploid perennial ryegrass mixture (consisting of 50% Barimero and 50% Toronto on a weight basis) was used as the control. The 2Nlow, 4Nlow and control grasses were grown at three N application levels (0, 12.5 or 25 g N m^{-2} $year^{-1}$), and the 2Nhigh and 4Nhigh grasses were grown at one N application level (25 g N m^{-2} $year^{-1}$) due to limited seed availability (Table 1). There were four replicates of each treatment. As a result, the experiment consisted of 44 different plots (5 × 2 m), in which treatments were allocated in a randomized block design. The experiment was established on a peat soil that had been in use as a permanent dairy grassland, with mean ditch water levels 60 cm below surface (KTC Zegveld, 52°08′N, 4°50′E). Two weeks before sowing, the field was treated with 0.25 ml m^{-2} Roundup® (480 g glyphosate l^{-1}, Monsanto, Saint Louis, MO, USA), and at the day before sowing, the terminated grass sod was rotavated to a depth of 10 cm. On 2 September 2016, grasses were sown at a rate of 1327 germinating seeds m^{-2}, which was approximately equal to 3 g m^{-2} for diploids and 4.6 g m^{-2} for tetraploids. These are common sowing rates in practice for diploid and tetraploid grasses (Remmelink *et al.*, 2018). The experiment was carried out until the first harvest in 2019. Each growing season, fields were fertilized with 0, 12.5 or 25 g N m^{-2} using calcium ammonium nitrate ($H_4CaN_2O_3$), 10 g K m^{-2} using potassium chloride (K_2SO_4) and 1.7 g P m^{-2} using

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Table 2. Experimental treatments of the pot and field experiments

		Pot experiment			Field experiment		
		N fertilization level ($g\,m^{-2}$)			N fertilization level ($g\,m^{-2}\,year^{-1}$)		
Population	Ploidy	0	6	12	0	125	250
1/2Nlow	2N	x	x	x	x	x	x
2	2N	x	x	x			
3	2N	x	x	x			
4	2N	x	x	x			
5	2N	x	x	x			
6	2N	x	x	x			
7	2N	x	x	x			
8	2N	x	x	x			
9/2Nhigh	2N	x	x	x			x
10/4Nlow	4N	x	x	x	x	x	x
11	4N	x	x	x			
12	4N	x	x	x			
13	4N	x	x	x			
14	4N	x	x	x			
15	4N	x	x	x			
16/4Nhigh	4N	x	x	x			x
Control	2N				x	x	x

Crosses (x) indicate treatment combinations of populations and N fertilization levels. Each treatment was replicated three times in the pot experiment and four times in the field experiment.

Table 3. Pot and field experiment

Soil parameter	Unit	Pot experiment	Field experiment
Organic matter	$g\,100\,g^{-1}$ dry soil	80	45.9
Clay	$g\,100\,g^{-1}$ dry soil	ND	31
Total C	$g\,kg^{-1}$ dry soil	408	232
Total N	$g\,kg^{-1}$ dry soil	5.6	21.1
P_{AL}	mg P_2O_5 $100\,g^{-1}$ dry soil	ND	22
Total K	$mg\,kg^{-1}$ dry soil	ND	484
Total S	$g\,kg^{-1}$ dry soil	2.0	6.0
pH-KCl	–	5.8	4.7

P_{AL}, ammonium lactate-acetate soluble phosphorus; pH-KCL, pH determined at a 1:5 soil to 1.0 M KCl solution ratio (ISO 10390:2005); ND, not determined.
Properties of the peat substrate used in the pot experiment and of the soil in the field experiment ($n = 2$).

Table 4. Field experiment

	Annual N fertilization level ($g\,m^{-2}$)	Applied N, K and P fertilizer per harvest cut ($g\,m^{-2}$)			
		End of March	After 1st cut	After 2nd cut	After 3rd cut
N	0.0	0.0	0.0	0.0	0.0
	12.5	5.0	3.5	2.5	1.5
	25.0	10.0	7.0	5.0	3.0
K	0.0–12.5–25.0	4.1	3.3	2.5	-
P	0.0–12.5–25.0	1.7	–	–	–

Scheme of annually applied nitrogen (N), potassium (K) and phosphorus (P).

monocalcium phosphate ($Ca(H_2PO_4)_2\ .H_2O$) (Table 4). The level and annual distribution of N fertilizer, at the treatment of 25 g $N\,m^{-2}\,year^{-1}$, was similar to local practice (Remmelink *et al.*, 2018).

Between May 2017 and May 2019, every 5–8 weeks aboveground herbage was harvested at a stubble height of approximately 5 cm using a small plot harvester, and weighed (J. Haldrup, Løgstør, Denmark). The harvesting regime was similar to local practice (Vellinga and André, 1999; Remmelink *et al.*, 2018). At each cut, a representative herbage sample was taken from each plot for analyses of DM (oven drying at 70°C for 48 h) and total shoot tissue N concentration (NEN-ISO 5983-2, Kjeldahl method, Eurofins Agro). Samples from the first cut in 2018 were analysed for WSC, neutral detergent fibre (NDF), acid detergent fibre (ADF) and *in vitro* digestibility of organic matter (IVDOM) using near-infrared spectroscopy (Eurofins Agro).

Soil samples (0–10 cm depth) of all plots were taken in February 2017. These were pooled by weight into one subsample, which was used for further analyses (Table 3). Average daily temperatures were collected from weather station de Bilt (Royal Netherlands Meteorological Institute, 52°05′N, 5°10′E), and precipitation data were collected from weather station Zegveld (Royal Netherlands Meteorological Institute, 52°07′N, 4°50′E)

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Table 5. Field experiment

	Temperature (°C)		Precipitation (mm)	
Period	Mean	S.D.	Sum	S.D.
Mar.–Oct. 2017	14.0	3.8	571	5
Mar.–Oct. 2018	14.6	5.0	337	3
Mar.–Oct. 1999–2019	13.6	4.9	587	5
Mar.–Apr. 2019	9.5	3.5	136	4
Mar.–Apr. 1999–2019	8.2	3.9	97	3

S.D., standard deviation.
Air temperatures and precipitation at the experimental site for each growing season.

(Table 5). These stations were located 23.6 and 2.5 km from the experimental site, respectively. Annual atmospheric N deposition (NO_x and NH_3) at the experimental site was estimated at 2.32 and 2.42 g m^{-2} in 2017 and 2018, respectively (Wichink Kruit and Van Pul, 2018) (data of 2019 were not available).

In May 2017 – the first spring after sowing – all fields received an additional calcium ammonium nitrate application of 2.5 g N m^{-2}, and all herbage was harvested at a stubble height of 7 cm and then discarded, in order to stimulate perennial ryegrass growth and tillering, and to decrease the presence of other spontaneously germinated plant species (mainly *Stellaria media*). The botanical composition of each plot was visually estimated in May 2017 and 2018.

Data analyses

The SNS in both experiments was determined as the shoot tissue N uptake at zero N fertilization (Hassink, 1995). The NUE was expressed by Eqn (1), in which ΔW is the increment of aboveground dry biomass weight between two N fertilization levels, and ΔNupt is the increment of shoot tissue N uptake between two N fertilization levels (Gastal *et al.*, 2015).

$$\mathrm{NUE} = \frac{\Delta \mathrm{W}}{\Delta \mathrm{Nupt}} \quad (1)$$

The NUptE was expressed by Eqn (2), in which ΔN supply is the fertilizer N increment between two N fertilization levels, assuming SNS remains constant at different N fertilization levels (Gastal *et al.*, 2015).

$$\mathrm{NUptE} = \frac{\Delta \mathrm{Nupt}}{\Delta \mathrm{Nsupply}} \quad (2)$$

Comparisons of the populations NUE and NUptE were done taking into account the nutritional N status, as suggested by Sandaña *et al.* (2021). They concluded that in order to seek for potential pathways to improve the NUE on grassland systems, both the nutritional N status and forage yield must be considered for the correct interpretation of NUE in response to genotype-nitrogen environmental conditions. We used the N nutrition index (NNI) as an assessment tool for the nutritional N status (Lemaire *et al.*, 2008). The NNI is expressed by Eqn (3), in which $\%N_a$ is the actual shoot tissue N concentration, and $\%N_c$ is the critical N concentration on a DM basis (Gastal *et al.*, 2015).

$$\mathrm{NNI} = \frac{\%\mathrm{N_a}}{\%\mathrm{N_c}} \quad (3)$$

The critical N concentration is considered to be the minimum plant N concentration needed for the maximum growth rate (Ulrich, 1952), which can be estimated via the critical N dilution curve. We estimated the critical N dilution curve for C3-grasses by the equation of Greenwood *et al.* (1991) (Eqn (4)), in which W is the aboveground biomass in Mg ha^{-1}.

$$\%\mathrm{N_a} = 4.8\,\mathrm{W}^{-0.32} \quad (4)$$

All statistical analyses were done using R (R Core Team, 2019). A P value ≤ 0.05 was considered significant at all analyses. Prior to the experiments, a power analysis was performed, based on the shoot tissue N concentration variance observed on sandy soil, to determine the minimum number of observations needed for a 90% probability ($\beta = 0.10$) that null hypotheses were not mistakenly accepted. In both experiments, analyses for differences between shoot tissue N concentration, NUE, NUptE, DM yield and fodder quality parameters were done with an ANOVA, in which population was used as factor, NNI (for NUE and NUptE) or N fertilization (for all other variables) was used as independent variable and replications were used as block effects. In the pot experiment, results for diploid and tetraploid populations were analysed separately, since previous recorded mean shoot tissue N concentrations on sandy soil were lower for diploids than for tetraploids. In the field experiment, results of the control, 2Nlow and 4Nlow populations at 0, 12.5 and 25 g m^{-2} N $year^{-1}$ fertilization, and results of all populations at 25 g m^{-2} N $year^{-1}$ fertilization, were analysed separately, since the design of the experiment was not fully balanced. In the field experiment, harvest number was used as a within-subject factor according to a repeated-measures design. Pots and plots were considered experimental units in the pot and field experiment, respectively. Differences among treatments were analysed by least significant differences. Correlation analyses between NUE and shoot tissue N concentration were done using Pearson correlation coefficients.

Results

Pot experiment

Shoot tissue N concentrations differed among tetraploid, but not among diploid populations (Table 6). Tetraploid population 10 had a significant lower shoot tissue N concentration compared to the other populations ($P < 0.001$). The NUE differed among diploid and tetraploid populations ($P = 0.008$ and $P = 0.005$, respectively). The NUptE differed among diploid ($P = 0.043$), but not among tetraploid populations. DM yields were affected by N fertilization ($P < 0.001$), but did not differ among populations.

Shoot tissue N concentrations of the tetraploid populations correlated negatively with NUE ($r = -0.85$ and $P = 0.014$) (Fig. 1). On average, shoot tissue N concentrations decreased 0.8 g kg^{-1} DM per g of NUE decrease among the tetraploid populations.

Within N fertilization levels, shoot tissue N concentrations differed among the diploid populations at 12 g m^{-2} N fertilization ($P = 0.005$), and DM yields did not differ among diploid and tetraploid populations (Table S2).

The mean SNS during the 20-day growing period was 2.7 ± 0.13 and 2.3 ± 0.11 g m^{-2} among diploid and tetraploid populations, respectively.

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Table 6. Pot experiment

Ploidy	Diploid					Tetraploid			
Parameter	Tissue N conc.	NUE	NUptE	DM yield	Parameter	Tissue N conc.	NUE	NUptE	DM yield
Unit	g kg^{-1} DM	g g^{-1} DM	g g^{-1} DM	g m^{-2}	Unit	g kg^{-1} DM	g g^{-1} DM	g g^{-1} DM	g m^{-2}
Population means					Population means				
1	30.7	27.4^{a}	0.43^{bc}	150	10	28.4^{a}	23.9^{a}	0.48	146
2	39.3	10.9^{d}	0.41^{bc}	134	11	36.0^{b}	16.3^{bc}	0.50	138
3	41.9	15.1^{cd}	0.38^{c}	123	12	35.1^{b}	17.1^{bc}	0.48	142
4	37.5	17.4^{bcd}	0.56^{ab}	140	13	36.9^{b}	12.6^{c}	0.48	136
5	37.9	15.1^{cd}	0.42^{bc}	131	14	37.0^{b}	15.3^{bc}	0.58	138
6	35.5	22.6^{ab}	0.42^{bc}	150	15	36.8^{b}	18.9^{ab}	0.51	147
7	35.6	16.1^{bcd}	0.62^{a}	151	16	38.5^{b}	15.2^{bc}	0.46	131
8	37.4	16.6^{bcd}	0.57^{ab}	105					
9	41.2	19.1^{bc}	0.42^{bc}	131					
S.E.M.	1.27	1.11	0.019	5.2	S.E.M.	1.42	0.92	0.017	5.3
Explained variance					Explained variance				
Population	3%ns	5%**	12%*	9%ns	Population	3%***	7%**	5%ns	2%ns
N fertilization	94%***			40%***	N fertilization	95%***			64%***
N nutrition index		94%***	78%***		N nutrition index		84%***	74%**	
Block	1%ns	0%ns	5%ns	1%ns	Block	2%ns	7%*	12%ns	4%*
Residual variation	5%	2%	5%	49%	Residual variation	1%	2%	8%	31%

ns, not significant.

Mean, standard error of the mean (S.E.M.) and explained variance of shoot tissue N concentration (tissue N conc.), N use efficiency (NUE), N uptake efficiency (NUptE) and dry matter (DM) yield for diploid and tetraploid populations.

^{abc}values with an unequal superscript differed significantly ($P < 0.05$).

***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

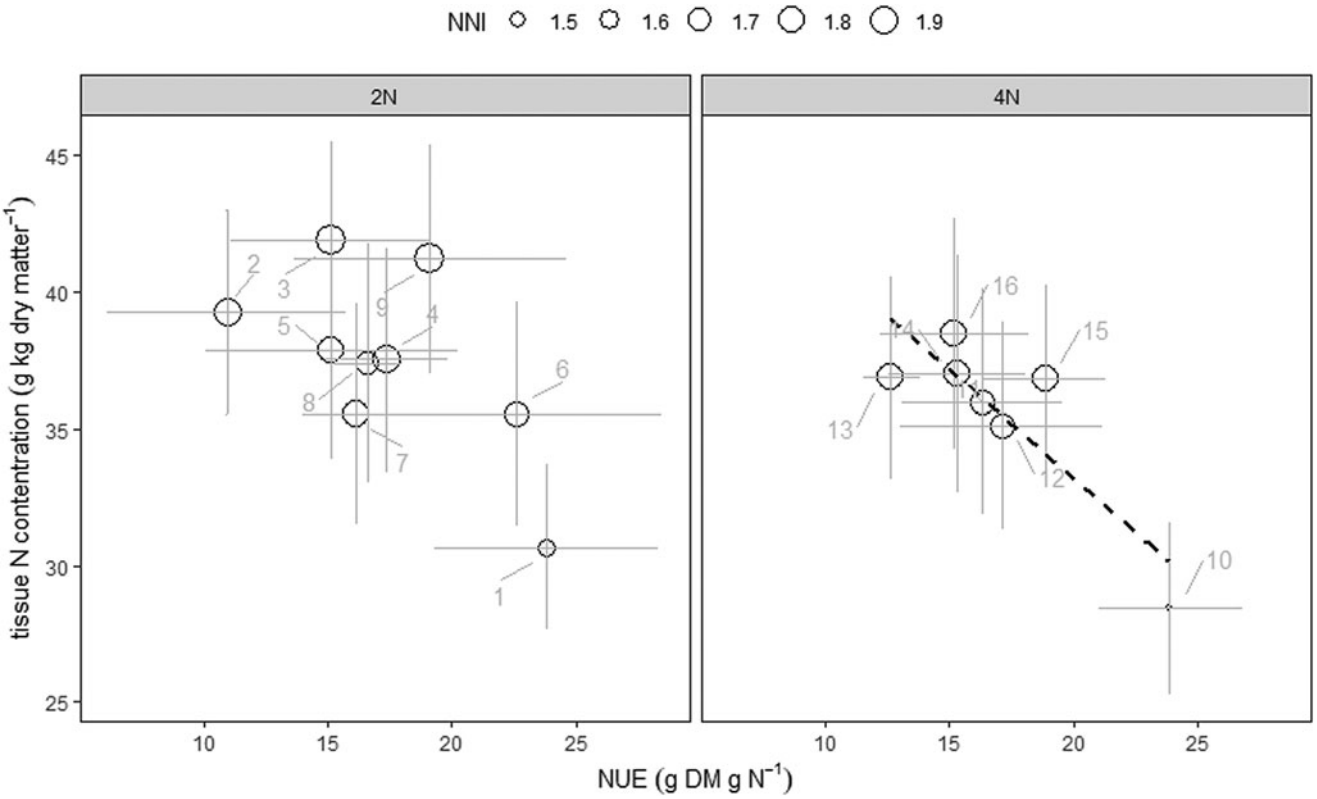

Figure 1. Pot experiment. Shoot tissue N concentration *v.* N use efficiency (NUE) of nine diploid (left; 2N) and seven tetraploid (right; 4N) perennial ryegrass populations. Symbol size reflects the N nutrition index (NNI). Horizontal and vertical bars represent standard errors of the mean. Numbers in the graphs indicate the perennial ryegrass populations. The dashed line represents the correlation between shoot tissue N concentration and NUE of the tetraploid populations ($r = -0.85$ and $P = 0.014$).

Field experiment: population effects at different N-fertilization levels

Grass shoot tissue N concentrations were affected by N fertilization ($P < 0.001$) and blocks ($P < 0.001$), but did not differ among the populations (Table 7). Shoot tissue N concentration variance was largely explained by N fertilization. The NUptE differed among populations ($P < 0.001$) and was affected by NNI ($P < 0.020$); the NUptE of population 4Nlow was lower than the control. The NUE was affected by NNI ($P = 0.001$), but did not differ among the populations. DM yields of 2Nlow were higher compared to 4Nlow ($P = 0.005$), and were affected by N fertilization ($P < 0.001$). At the first harvest of 2019, NDF differed among populations ($P = 0.038$) and was affected by N fertilization ($P < 0.001$). Concentrations of WSC and ADF and the percentage of IVDOM did not differ among populations, at the first harvest of 2019 (Table S3).

Field experiment: effects of the low and high-N populations at 25 g m^{-2} year^{-1} N fertilization

Populations 2Nlow and 2Nhigh had a lower shoot tissue N concentration than the control ($P = 0.018$) (Table 8). Population 2Nlow had a higher DM yield than population 4Nhigh ($P = 0.032$). At the first harvest of 2019, population 4Nhigh had a lower NDF than the control, populations 2Nlow and 4Nlow ($P = 0.015$). The WSC, ADF and percentage of IVDOM did not differ among the populations at the first harvest of 2019 (Table S4).

Field experiment: effects within harvests

Mean shoot tissue N concentrations of the control, 2Nlow and 4Nlow populations at 0, 12.5 and 25 g m^{-2} year^{-1} N fertilization differed at the first harvests of 2017, 2018 and 2019, and at the second harvest of 2018 (Figs 2 and S1). Shoot tissue N concentrations of the control and 2Nlow were lower compared to 4Nlow at the first harvests of 2017 and 2019 ($P < 0.001$ and $P = 0.003$, respectively), and shoot tissue N concentrations of 2Nlow were lower compared to the control and 4Nlow at the first and second harvest of 2018 ($P < 0.001$ and $P = 0.015$, respectively).

Mean shoot tissue N concentrations of the control, 2Nlow, 4Nlow, 2Nhigh and 4Nhigh populations at 25 g m^{-2} year^{-1} N fertilization differed across all harvests, except at the fourth harvest in 2017, and at the third and fourth harvest in 2018 (Figs 2 and S2). Differences among population shoot tissue N concentrations were inconsistent among harvests, at 25 g m^{-2} year^{-1} N fertilization.

Within harvests, NUE did not differ among the control, 2Nlow and 4Nlow populations, except at the second harvest in 2017 where 2Nlow and 4Nlow had a higher NUE than the control ($P = 0.031$) (Table S5). The NUptE differed among populations at the third harvest in 2017 ($P < 0.001$), at the first and second harvest in 2018 ($P = 0.003$ and $P = 0.001$, respectively) and at the first harvest in 2019 ($P = 0.001$). At these harvests, either 2Nlow, 4Nlow or both had a lower NUptE than the control. Lower shoot tissue N concentrations did not coincide with lower NUptEs at the first and second harvest in 2018 and at the first harvest in 2019.

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Table 7. Field experiment

Parameter	Shoot tissue N concentration	NUE	NUptE	DM yield	NDF
Unit	g kg^{-1} DM	g g^{-1} DM	g g^{-1} DM	g m^{-2}	g kg^{-1} DM
Population means					
Control	30.7	22.7	0.41^{a}	235^{ab}	495^{ab}
2Nlow	30.3	23.1	0.33^{ab}	241^{a}	497^{a}
4Nlow	30.7	25.2	0.26^{b}	228^{b}	487^{b}
S.E.M.	0.26	1.17	0.039	4.6	2.9
Explained variance					
Population	0%	3%ns	44%***	8%**	6%*
N fertilization	90%***			74%***	64%***
N nutrition index		43%**	12%*		
Block	6%***	5%ns	11%ns	1%	5%ns
Residual variation	4%	49%	33%	17%	25%

ns, not significant.
Mean, standard error of the mean (S.E.M.) and explained variance of shoot tissue N concentration, N use efficiency (NUE), N uptake efficiency (NUptE), dry matter (DM) yield and neutral detergent fibre (NDF) of the control, 2Nlow and 4Nlow populations at all N fertilization levels. Shoot tissue N concentration, NUE, NUptE and DM yield were determined across ten consecutive harvests, and NDF was determined at the first harvest of 2019 only.
^{abc}values with an unequal superscript differed significantly ($P < 0.05$).
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

Table 8. Field experiment

Parameter	Shoot tissue N concentration	DM yield	NDF
Unit	g kg^{-1} DM	g m^{-2}	g kg^{-1} DM
Population means			
Control	35.0^{a}	252^{ab}	511^{ab}
2Nlow	33.6^{b}	260^{a}	516^{a}
4Nlow	34.4^{ab}	243^{ab}	506^{ab}
2Nhigh	33.6^{b}	243^{ab}	494^{bc}
4Nhigh	34.7^{ab}	238^{b}	487^{c}
S.E.M.	0.29	7.3	3.3
Explained variance			
Population	36%*	56%*	56%*
Block	40%**	0%ns	4%ns
Residual variation	24%	44%	41%

ns, not significant.
Mean, standard error of the mean (S.E.M.) and explained variance of shoot tissue N concentration, dry matter (DM) yield and neutral detergent fibre (NDF) of the control, 2Nlow, 2Nhigh, 4Nlow and 4Nhigh populations at a 25 g m^{-2} year^{-1} N fertilization level. Shoot tissue N concentration and DM yield were determined across ten consecutive harvests, and NDF was determined at the first harvest of 2019 only.
^{abc}values with an unequal superscript differed significantly ($P < 0.05$).
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

At the second harvest in 2018, DM yields were similar among fertilizer N levels. At the third and fourth harvest in 2018, DM yields were higher in unfertilized than in fertilized conditions (Fig. 2).

Field experiment: soil nitrogen supply, estimated soil coverage and weather conditions

The mean annual SNS in 2017 and 2018 was 26.1 ± 1.2 g N m^{-2}, and the mean SNS until the first harvest of 2019 was 6.0 ± 0.7 g N m^{-2} (Table S6). Estimated shares of soil coverage by perennial ryegrass were 88.9 ± 3.6% and 86.8 ± 4.9% in May 2017 and 2018, respectively, and did not differ among the populations (Table S7). Growing season temperatures at the experimental site were higher than 20-year averages, and the 2018 growing season was relatively dry, having the fifth lowest recorded precipitation deficit since 1901 (Sluijter *et al.*, 2018).

Discussion

The first hypothesis, that perennial ryegrass populations selected for different shoot tissue N concentrations on a sandy soil have different shoot tissue N concentrations, NUE or NUptE when grown on a peat soil, was confirmed in the pot and field experiments, although results were not fully consistent between the experiments. The second hypothesis, that populations with a higher NUE will have lower shoot tissue N concentrations, was confirmed among the tetraploid populations but not among the diploid populations in the pot experiment, and not in the field experiment. The third hypothesis, that populations with a lower NUptE have lower shoot tissue N concentrations, was neither confirmed in the pot nor in the field experiment.

Population differences in shoot tissue N concentration

Mean shoot tissue N concentrations were higher in the pot experiment (36.6 g kg^{-1} DM) and similar in the field experiment (31.3 g kg^{-1} DM), compared to mean values of 60 years of Dutch grassland experiments on peat soil (about 29–30 g kg^{-1} DM) (Vellinga and Andre, 1999). Mean SNS, expressed on a daily basis, was 0.12 g m^{-2} in the pot experiment, 0.11 g m^{-2} in the field experiment at the growing seasons of 2017 and 2018 and 0.09 g m^{-2} until the first harvest of 2019 (29 April), respectively, and was within the range of previously observed mean estimations of SNS for dairy grasslands in the western peat area of the Netherlands (0.11–0.13 g m^{-2} day^{-1} at an average growing season and 0.08–0.12 g

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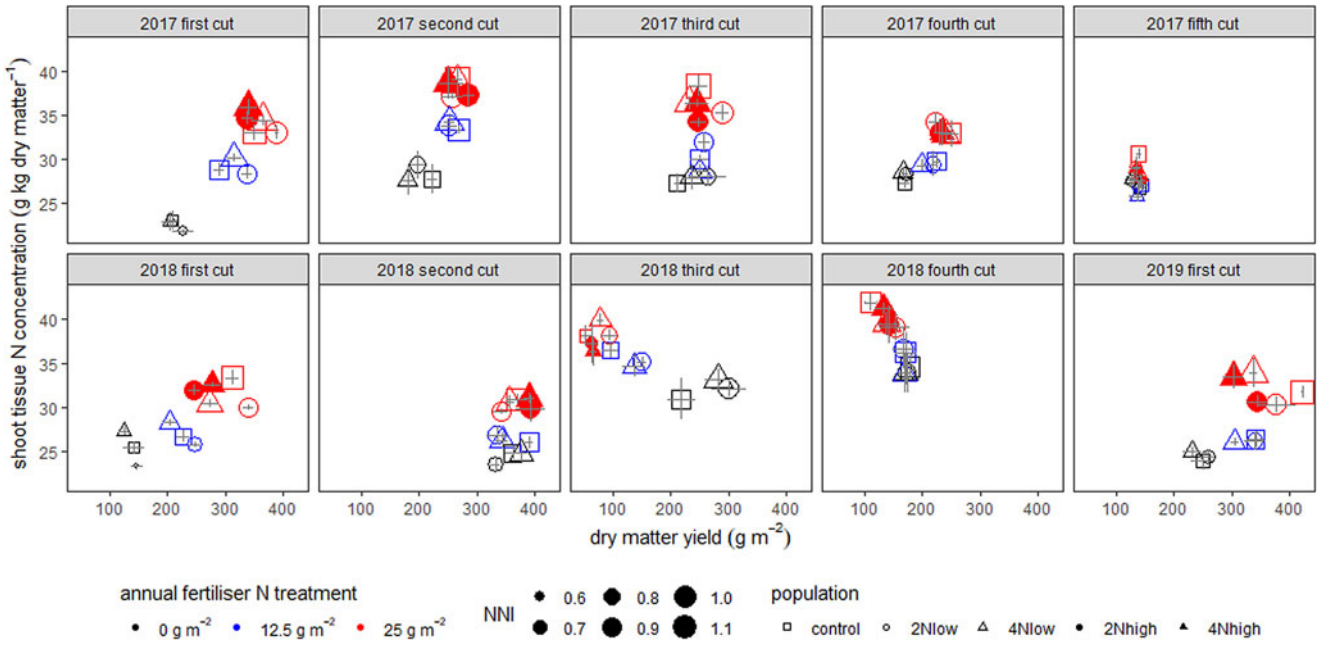

Figure 2. Field experiment. Relationship between shoot tissue N concentration and dry matter yield, per population, N fertilization level and harvest cut. Symbol size reflects the N nutrition index (NNI). Horizontal and vertical bars represent standard errors of the mean.

m^{-2} day^{-1} on average until 29 April, at soil organic matter contents of 35–55 g 100 g^{-1} dry soil, respectively) (Vellinga and Andre, 1999; Pijlman *et al.*, 2020b).

Differences in shoot tissue N concentration among populations were small compared to the effect of N fertilization in both experiments, as expected (Wilkins and Lovatt, 1989; Malmberg *et al.*, 2023). In the pot experiment, only the tissue N concentration of population 10 was lower compared to the other tetraploid populations, while in the field experiment the shoot tissue N concentration of population 10 (renamed to 4Nlow) was not different from tetraploid population 4Nhigh. In the pot experiment, population 10 also had a high NUE and low NNI (1.41 *v.* 1.79, respectively) compared to the other tetraploid populations. The low NNI indicated that population 10 had a lower luxury N consumption compared to the other populations (Lemaire *et al.*, 2008). The NNI of population 10 (4Nlow) was also low compared to population 4Nhigh in the field experiment (0.82 and 0.93, respectively), in line with the results of the pot experiment.

Population differences in N use efficiency and N uptake efficiency

The nutritional N status explained a larger part of the NUE and NUptE variance than the populations, in both experiments. This is in line with other studies, in which N fertilization – which greatly affects the nutritional N status of plants (Lemaire *et al.*, 2008) – had a greater effect than the genetic component on shoot tissue N concentration (Wilkins and Lovatt, 1989; Radojevic *et al.*, 1994; Sampoux *et al.*, 2011; Robins and Lovatt, 2016). However, these studies were carried out on mineral soils, and not all of these studies were specifically carried out to assess the effects of genetic variation on shoot tissue N concentration at different N fertilization levels.

Differences in NUptE were relatively greater than differences in shoot tissue N concentration among the diploid populations in the pot experiment and in the field experiment, similar to the results of Wilkins and Lovatt (1989), who tested four perennial ryegrass varieties at three different N fertilization levels in a field experiment on mineral soil. They reported 18 and 6% variation among varieties in N recovered to N applied and shoot tissue N concentration, respectively.

Relationship between shoot tissue N concentration, N use efficiency and N uptake efficiency

The negative correlation between NUE and shoot tissue N concentrations among the tetraploid populations in the pot experiment is in line with the results of earlier studies (Wilkins *et al.*, 2000; Sandaña *et al.*, 2021). However, the effect of NUE on shoot tissue N concentration was largely due to population 10, which had a lower tissue N concentration and a higher NUE compared to the other tetraploid populations (with exception of population 15 of which the NUE did not differ from population 10). Since NUE and shoot tissue N concentrations differed among the tetraploid populations in the pot experiment, it is likely that the selection of perennial ryegrass with a higher NUE will result in genotypes with a lower shoot tissue N concentration per unit of DM gain. In different studies, it was hypothesized that the negative relationship between NUE and shoot tissue N concentrations could be the result of a DM 'dilution' effect, caused by genetic differences in efficiency of protein synthesis and turnover (Wilkins *et al.*, 2000; Baert *et al.*, 2007). According to this hypothesis, reducing the shoot tissue N concentration via breeding can lead to increased DM yields at a similar shoot tissue N uptake (Wilkins and Lovatt, 1989; Baert *et al.*, 2007). Our results suggest that there was no correlation between NUE and DM yield among the tested populations. In the pot experiment, DM yields did not differ among populations, while there were differences in shoot tissue N concentration and NUE among the tetraploid populations. In the field experiment, across the ten harvests, DM yields differed among populations, but no differences in NUE among

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populations were found. Moreover, in the field experiment, at an N fertilization rate of 25 g m^{-2} $year^{-1}$ and across the ten harvests, shoot tissue N concentrations and DM yields differed among populations, but there was no correlation between shoot tissue N concentrations and DM yields.

Our results suggest that there was no relationship between NUptE and shoot tissue N concentration in the pot and the field experiment. In the pot experiment, among the diploid populations, NUptE differed but shoot tissue N concentrations did not, suggesting that NUptE did not affect shoot tissue N concentration. In the field experiment, NUptE and shoot tissue N concentrations differed among populations at the first and second harvest of 2018 and at the first harvest of 2019, but lower shoot tissue N concentrations did not coincide with lower NUptEs at these harvests. However, we cannot exclude that genetic variation of shoot tissue N concentration and NUptE could have been masked by genotype–environmental interactions. For example, the ranking of shoot tissue N concentrations among the populations was not fully consistent between the experiments, and with concentrations recorded on the sandy soil. Genotype by environment interactions have been observed in several studies. Robins and Lovatt (2016) showed that genotype by environment interactions can affect the feeding value of perennial ryegrass, of which shoot tissue N concentration is a component, at three sites on mineral soils differing in water availability, climate and altitude. Parsons *et al.* (2011) pointed out that genotype by environment interactions affect trait expressions, and that the 'success' of traits such as high WSC or 'low respiration' largely depends on the N fertilization rate. This means that further research is needed to confirm that NUptE is not correlated to the shoot tissue N concentration of perennial ryegrass, grown at dairy grasslands on peat soil.

Within harvest effects in the field experiment

Differences in shoot tissue N concentration among populations were inconsistent within harvests and years, in the field experiment. Inconsistency of shoot tissue N concentrations among populations was possibly a result of varying environmental conditions. In the field experiment, the greatest differences in shoot tissue N concentration were observed in the early harvest cuts within a season, and differences in shoot tissue N concentration among fertilization levels were greater at the first cuts of the growing season, compared to the last cuts. This may have been the result of a combination of a higher growth rate of grass in spring compared to later in the growing season – which results in a lower soil N availability per unit of DM growth (Davies, 1971; Harris *et al.*, 1996; Burns *et al.*, 2012) – and of a lower SNS in spring compared to summer and autumn (Vellinga and André, 1999; Pijlman *et al.*, 2020b), although N fertilization rates decreased with every cut.

At the second harvest in 2018 in the field experiment, DM yields were similar among fertilizer N levels, and from the third harvest onwards, DM yields were lower in fertilized compared to unfertilized treatments. The year 2018 had a relative dry growing season. After the first harvest of 2018, soil moisture likely had a greater influence on grass regrowth and N uptake than soil mineral N availability, specifically at the N fertilized treatments which produced relatively high DM yields before dry conditions arrived (Gonzalez-Dugo *et al.*, 2010). The significant effect of blocks on shoot tissue N concentration was possibly also related to the weather conditions, which may have increased the spatial and spatio-temporal variation in soil moisture and SNS (Wang *et al.*, 2021). At the second harvest in 2017 and the fourth harvest in 2018, the largest differences in shoot tissue N concentration were observed among blocks (data not shown), and both of these harvests were carried out during dry periods. Variation in weather conditions and soil moisture can directly or indirectly affect grass N uptake by influencing soil moisture or SNS (Vellinga and André, 1999; Gonzalez-Dugo *et al.*, 2010; Sampoux *et al.*, 2011; Pijlman *et al.*, 2020b). The dry growing season of 2018 may also have reduced differences among populations regarding N uptake and use, because N-rich plant organs are more sensitive to water deficit than N-poor organs (Gonzalez-Dugo *et al.*, 2010).

The lower NDF of population 4Nlow compared to 2Nlow across all N fertilization levels was likely an effect of ploidy. Tetraploids are generally leafier and have larger cells and a better digestibility than diploids, resulting in a lower NDF (Burns *et al.*, 2012; Griffiths *et al.*, 2017). This difference in shoot tissue NDF concentration between populations indicated that, after 3 years including the very dry growing season of 2018, the sown populations were still dominant in the experimental plots.

Limitations to the current study

There were a few limitations to the current study. By using the diploid and tetraploid populations in the field experiment, which had either the lowest or highest recorded shoot tissue N concentration in the pot experiment, it was assumed that the population variation on shoot tissue N concentration was similar in the pot and field experiment, despite differences in environment and moisture availability which could have affected genotype expressions (Robins and Lovatt, 2016). Shoot tissue N uptakes were indeed higher in the pot compared to the field experiment, in line with Poorter *et al.* (2016), who concluded that plants grown inside grow faster, take up more N and may differ in morphology, compared to plants grown in field conditions.

Furthermore, there were differences between the peat-based substrate used in the pot experiment, and the peat soil at which the field experiment was carried out, although in both experiments, P, K and S were considered not to be limiting grass growth (CBGV, 2022). The peat-based substrate mix had a higher organic matter content and pH, compared to the soil of the field experiment, which may have resulted in a higher N availability in the pot experiment (Egan *et al.*, 2019). It is also likely that the substrate and field soil differed in physical properties, due to differences in origin (Loisel *et al.*, 2014) and history of use of the peat (Kechavarzi *et al.*, 2010). These differences between the pot and field experiment may have influenced the expression of the genetic variation in N uptake, NUE and NUptE of the populations (Parsons *et al.*, 2011; Robins and Lovatt, 2016), which means that results from the pot experiment may not be completely transferable to field conditions.

The selection of populations grown on a sandy soil was only based on shoot tissue N concentration, without taking into account NNI, NUE, NUptE or morphological traits such as heading date or maturity, which may have affected the population selection for the experiments (Gastal *et al.*, 2015; Sandaña *et al.*, 2021). Furthermore, selection of perennial ryegrass populations for the pot experiment was based on data of first three harvests, and selection of populations for the field experiment was based on a single May harvest 62 days after sowing. This could have favoured populations differing in shoot tissue N concentration mostly at the beginning of the growing season. Possibly, selection of populations with increased summer and/or autumn growth

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may result in N concentrations better matching the annual SNS pattern. However, to our knowledge, there is only limited evidence of a relationship between maturity or heading date, and shoot tissue N concentration of perennial ryegrass (Sampoux *et al.*, 2011; Wilkins and Lovatt, 2011; Burns *et al.*, 2012). Only Burns *et al.* (2012) found that later maturity was associated with higher annual herbage yields and shoot tissue N concentrations, on a clay loam soil, but this effect was not expressed in each seasonal period. Future selection experiments should therefore cover full season research across a range of weather conditions, to select for populations with consistently lower mean annual shoot tissue N concentrations.

Conclusions

For perennial ryegrass populations selected for different shoot tissue N concentrations using a sandy soil, the pot experiment using a peat substrate showed that, among tetraploid populations, lower shoot tissue N concentrations coincided with a higher NUE, and that among diploid populations, NUptE but not shoot tissue N concentrations differed. The field experiment on peat soil showed that populations could explain 44% of the variation in NUptE, but no relation between NUE or NUptE and shoot tissue N concentration was found. Across ten harvests at an N fertilization level close to local practice, selected populations had a 1.4 g kg^{-1} DM lower shoot tissue N concentration, but not a different DM yield, compared to a commercial control. The results show potential for the selection of perennial ryegrass populations with low shoot tissue N concentration for dairy grassland on peat soil, by selecting for shoot tissue N concentration and NUE, and show potential for the selection of populations for NUptE.

Supplemental material. This manuscript includes seven supplementary tables and two supplementary figures; Table S1 on the air temperatures and precipitation near the commercial breeding site on a sandy soil; Table S2 on shoot tissue N concentration and DM yields within N fertilization levels in the pot experiment; Tables S3 and S4 on the shoot tissue WSC and ADF concentrations, and percentage of *in vitro* digestible organic matter of the populations in the field experiment; Fig. S1 on mean shoot tissue N concentrations of the control, 2Nlow and 4Nlow populations per harvest at all N fertilization levels in the field experiment; Fig. S2 on mean shoot tissue N concentrations of all tested populations per harvest at the 25 g N m^{-2} $year^{-1}$ fertilization level in the field experiment; Table S5 on NUE and NUptE per population and harvest in the field experiment; Table S6 on N uptakes per population and N fertilization level in the field experiment; and Table S7 on the visually estimated soil coverage by perennial ryegrass in the field experiment.

Suplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0021859623000394

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Author contributions. N. H., J. D. and N. v. E. conceived and designed the study. J. P., N. H. and J. D. conducted data gathering and performed statistical analyses. J. P., N. H., J. D., J. W. E. and N. v. E. wrote the article.

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Competing interest. None.

Ethical standards. Not applicable.

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