Grass and Forage Science
The Journal of the British Grassland Society | The Official Journal of the European Grassland Federation

Species interactions in a grassland mixture under low nitrogen fertilization and two cutting frequencies. II. Nutritional quality

Å. Ergon*, L. Kirwan†, G. Fystro‡, M. A. Bleken§, R. P. Collins¶ and O. A. Rognli*

*Department of Plant Sciences, Norwegian University of Life Sciences, Ås, Norway, †UCD Institute of Food and Health, Science Centre South, University College Dublin, Dublin 4, Ireland, ‡Norwegian Institute of Bioeconomy Research, Ås, Norway, §Department of Environmental Sciences, Norwegian University of Life Sciences, Ås, Norway, ¶Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Aberystwyth, UK

Abstract

Mixtures and pure stands of perennial ryegrass, tall fescue, white clover and red clover were grown in a three-cut and a five-cut system in southern Norway, at a low fertilization rate (100 kg N ha^{-1} year^{-1}). The nutritional quality (annual weighted averages) of the dried forage from the two-first harvesting years was analysed. There was no significant effect of species diversity on crude protein (CP) concentration. In the three-cut system, we found a significant species diversity effect leading to 10% higher concentrations of acid detergent fibre (ADF), 20–22% lower concentrations of water-soluble carbohydrate (WSC) and 4% lower net energy for lactation (NE_L) concentrations in mixtures compared with pure stands (averaged across the two-first years). In the five-cut system, similar effects were seen in the first year only. This diversity effect was associated with a reduction in WSC and NE_L concentrations and an increase in ADF, NDF and CP concentrations in the grass species, and not in red clover, when grown in mixtures. This is thought to be a combined result of better N availability and more shading in the mixtures. Species diversity reduced the intra-annual variability in nutritional quality in both cutting systems.

Keywords: *Festuca arundinacea*, forage quality, *Lolium perenne*, species diversity, *Trifolium pratense*, *Trifolium repens*

Correspondence to: Å. Ergon, Department of Plant Sciences, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway.
E-mail: ashild.ergon@nmbu.no

Received 23 October 2015; revised 5 July 2016

Introduction

There are marked differences in the nutritional composition of grasses and legumes, with legumes having a higher concentration of crude protein (CP) and a lower concentration of fibre than grasses (e.g. Hatfield *et al.*, 2007). The digestibility of all forages is reduced as a consequence of reproductive development due to an increase in the proportion of stems and lignified cellulose, accompanied with an increase in fibre concentration and a decrease in CP and readily digestible carbohydrates. The rate of decrease in digestibility with reproductive development tends to be lower in legumes than in grasses (Moore and Jung, 2001). There are also differences among species of legumes and grasses, which are partly a result of variations in growth habit and the allocation of resources between stem and leaf tissues. The timing of reproductive development and stem elongation varies among and within species, as does the tendency to produce stems during regrowth after defoliation. As a result of the strong effect of heading, stem development and the age of tissues (Duru, 2008), nutritional composition is highly influenced by the defoliation regime, with earlier and more frequent defoliation resulting in forage of higher digestibility (Gardarin *et al.*, 2014).

The inclusion of legumes in grassland swards has several advantages, such as providing N to the grasses through symbiotic N fixation, contributing to the dry-matter (DM) overyielding which is often obtained in species mixtures, and increasing the voluntary intake of forage by livestock due to attributes that increase the rate of passage through the rumen [reviewed by Lüscher *et al.* (2014)]. Nutritional quality is also more stable across harvests in grass–legume mixtures than in pure stands of grasses (Sleugh *et al.*, 2000; Sanderson, 2010). This is partly because grasses are generally earlier in reproductive development than legumes, resulting in the effects of reproductive development being distributed over a larger time span and balanced by the presence of species at other developmental stages. The nutritional quality of mixtures is largely determined by the dominant species in the mixture, and therefore, it may change as the species composition changes from year to year (Deak *et al.*, 2007; Sturludottir *et al.*, 2014; Brink *et al.*, 2015). There are few reported examples of species diversity effects independent of sampling effects occurring among the species in a sward. Species diversity may also improve nutritional quality through the repression of weeds (Tracy *et al.*, 2004; Picasso *et al.*, 2008) if these have lower nutritional value. There is limited information available on how the nutritional composition of forage plants is affected by plant interactions through N fixation or competition for light, water and nutrients. We conducted an experiment with a four-species mixture sown with variable species proportions and cultivated at a low N input level and in two different cutting systems. The effects of species diversity and cutting frequency on DM yield and changes in botanical composition over time in the same experiment were described by Ergon *et al.* (2016). Here, we analysed the effects of species diversity on the swards' nutritional quality and intra-annual stability over the two-first harvesting years. We asked the following questions:

1. Are there species diversity effects on forage nutritional variables?
2. Are any such diversity effects affected by cutting frequency?
3. What effect does species diversity have on intra-annual stability of nutritional quality?

Materials and methods

Field experiment

A field experiment, described in detail in Ergon *et al.* (2016), was conducted at Ås, Norway. In brief, pure stands and mixtures of perennial ryegrass (*Lolium perenne* L.), tall fescue (*Festuca arundinacea* Schreb.), red clover (*Trifolium pratense* L.) and white clover (*Trifolium repens* L.) were sown in 2010 in a split-plot design with cutting system (three or five cuts per year) as main plots, and sward type as subplots. Fertilizer was applied at a rate equal to 100 kg N ha^{-1} year^{-1}. After each harvest in 2011 and 2012, the harvested material within each plot was mixed, and a sample (approximately 1 kg) was taken from all plots of one of the two seed-rate treatments (20 kg ha^{-1}) in the experiment. In order to study the effect of sward type on nutritional variables of single species, samples sorted into species fractions were also taken at the first and last harvest in the three-cut system in 2012.

Analysis of nutritional quality

Samples were dried at 60°C and cut into smaller pieces. Representative subsamples were milled in a Cyclotec 1093 sample mill (Foss A/S, Hillerød, Denmark) with a 1-mm sieve, and scanned with an NIR spectrophotometer (NIRSystems 6500, Silver Spring MD, USA). The content of crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), non-fibre carbohydrates (NFC), water-soluble carbohydrates (WSC), net energy for lactation (NE_L), digestible energy (DE) and metabolizable energy (ME) were determined in approximately 20% of the samples by chemical analyses of CP, NDF, ADF, NFC and WSC and calculations based on these variables, at Dairy One Forage Testing Laboratory, Ithaca, NY USA. Energy variables were estimated according to Weiss *et al.* (1992), Van Soest and Fox (1992), Weiss (1993, 1995). NIR data analysis, including collection of spectra, selection of samples for chemical analysis, local calibration and prediction, was conducted using ISI software (NIRS2, ver. 4·00, Intrasoft International, Silver Spring, MD, USA). Calibration and validation statistics are shown in a supplementary file (Table S1 of Supporting Information in the online version of this paper).

Statistical analysis

For each of the nutritional components, the weighted average concentration across harvests,

$$\sum_{i=1}^{h}\left(\text{concentration}_i \times \frac{\text{dry matter yield}_i}{\text{dry matter yield}_{\text{total}}}\right),$$

and the annual yield of the nutritional component,

$$\sum_{i=1}^{h}(\text{concentration}_i \times \text{dry matter yield}_i),$$

where i is the harvest number and h is the total number of harvests per year, were calculated for each plot and year. The average values for each year and treatment are shown in a supplementary file (Table S2 of Supporting Information). The effect of cutting system, the species identity effects β_{Species}, the species diversity effect δ and the contributions of the pairwise species interactions $\delta_{\text{Species 1 * Species 2}}$ to δ were estimated using the diversity-interaction models developed by Kirwan *et al.* (2007, 2009). This was performed as in Ergon *et al.* (2016, Model 1), using weighted average concentrations and annual yield of nutritional components as Y. The estimated species and species interaction coefficients were used to estimate the effect of varying the species composition of the four-species seed mixture on NE_L yield, keeping the proportion of each species within the 0·1–0·7 range. The intra-annual stability of nutritional quality was assessed using a mixed models approach to estimate the variability of the responses across harvests within a year. This was also performed as described in Ergon *et al.* (2016, Model 2). All models were fitted using the GLM and MIXED procedures in SAS 9·2 (SAS Institute Inc., Cary, NC, USA).

Results

Species diversity effects on nutritional composition of harvested forage

There were typical differences between legume and grass pure stands with legumes having higher concentrations of CP (163–2260 g kg^{-1} DM) and lower concentrations of NDF (342–438 g kg^{-1} DM) and WSC (136–187 g kg^{-1} DM) than grasses (90–130 g kg^{-1} DM, 453–544 g kg^{-1} DM and 232–282 g kg^{-1} DM respectively) (β coefficients in Table 1). The legume pure stands tended to have higher NE_L concentrations (5·89–6·31 MJ kg^{-1} DM) than grass pure stands (5·53–6·11 MJ kg^{-1} DM).

There was a significant diversity effect on ADF, WSC, NFC and NE_L concentrations in both cutting systems except in the five-cut system in the second year (Table 1). There was also a significant diversity effect on NDF concentration in the five-cut system in the first year. The effect was positive for NDF and ADF, leading to concentrations in the centroid mixture which was 7–10% higher than in the average pure stand (Table 2). For WSC, NFC and NE_L, the diversity effect was negative, leading to a WSC concentration in a centroid mixture which was 20–22% lower, a NFC concentration which was 7–9% lower, and a NE_L concentration which was 4% lower than in the average pure stand. There was no significant species diversity effect on the CP concentration (Table 1). The diversity effects were not always transgressive (i.e. having a higher value than the highest pure stand value) and it was therefore checked whether the observed diversity

Table 1 Parameter estimates for species identity coefficients β (Lp, *Lolium perenne*; Fa, *Festuca arundinacea*; Tr, *Trifolium repens*; Tp, *Trifolium pratense*) and the species diversity coefficient δ, on nutritional variables in mixtures of the four species. The species identity coefficients equal the variable estimates in pure stands (g kg^{-1} DM or MJ kg^{-1} DM), while the species diversity coefficient equals the variable estimate in a centroid mixture minus the average of pure stands.

Cutting system	Year	Parameter	Nutritional variable					
			CP	NDF	ADF	WSC	NFC	NE_L
Three-cut	Year 1	β_{Lp}	**103**	**488**	**280**	**282**	**326**	**5·94**
		β_{Fa}	**95**	**544**	**320**	**232**	**275**	**5·53**
		β_{Tr}	**214**	**375**	**290**	**144**	**317**	**6·06**
		β_{Tp}	**199**	**380**	**307**	**136**	**322**	**5·91**
		δ	5	24	**28**	**−40**	**−25**	**−0·23**
	Year 2	β_{Lp}	**109**	**507**	**301**	**246**	**305**	**5·80**
		β_{Fa}	**90**	**508**	**289**	**277**	**309**	**5·81**
		β_{Tr}	**163**	**438**	**293**	**187**	**307**	**5·93**
		β_{Tp}	**178**	**395**	**305**	**170**	**325**	**5·89**
		δ	15	9	**28**	**−50**	**−21**	**−0·19**
Five-cut	Year 1	β_{Lp}	**109**	**507**	**302**	**241**	**298**	**5·79**
		β_{Fa}	**115**	**515**	**301**	**238**	**290**	**5·75**
		β_{Tr}	**226**	**369**	**276**	**141**	**320**	**6·23**
		β_{Tp}	**204**	**363**	**291**	**149**	**338**	**6·12**
		δ	−4	**43**	**23**	**−44**	**−28**	**−0·23**
	Year 2	β_{Lp}	**130**	**453**	**266**	**259**	**329**	**6·11**
		β_{Fa}	**112**	**494**	**285**	**255**	**302**	**5·86**
		β_{Tr}	**213**	**374**	**269**	**147**	**323**	**6·24**
		β_{Tp}	**206**	**342**	**252**	**158**	**352**	**6·31**
		δ	9	4	−2	−13	−7	0·00

Estimates significant at $P < 0·05$ are in bold type.

CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water-soluble carbohydrates; NFC, non-fibre carbohydrates; NE_L, net energy for lactation.

Table 2 Species diversity effects on nutritive components in a centroid mixture predicted from Model 1 and expressed as per cent change relative to the value expected from the sown species proportions and the values of pure stands. Significance levels of the diversity effect estimates are given.

	Three-cut system		Five-cut system	
Nutritional variable	Year 1	Year 2	Year 1	Year 2
NDF (% of DM)	NS	NS	9*	NS
ADF (% of DM)	10***	10***	7**	NS
WSC (% of DM)	−20**	−22**	−21**	NS
NFC (% of DM)	−8**	**−7***	**−9*****	NS
NE_L (MJ kg^{-1} DM)	−4**	**−4****	−4**	NS
CP yield (t ha^{-1})	**76*****	**83*****	**60*****	**68****
NE_L yield (GJ ha^{-1})	**52*****	**56*****	**68*****	**72*****
DM yield (t ha^{-1})	**56*****	**90*****	**64*****	**94*****

Transgressive diversity effects are in bold type.
NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water-soluble carbohydrates; NFC, non-fibre carbohydrates; NE_L, net energy for lactation; DM, dry matter.
***$P < 0{\cdot}0001$; **$0{\cdot}0001 < P < 0{\cdot}005$; *$0{\cdot}005 < P < 0{\cdot}05$.

effects could be due to changes in species composition relative to the sown proportions (a sampling effect) by comparing the measured ADF, NDF, WSC, NCF and NE_L concentrations with the concentrations that would be expected based on observed, rather than sown, species proportions in the mixtures (Table 3). The measured concentrations of ADF, NDF, WSC and NFC concentrations in both cutting systems, and of NE_L concentration in the three-cut system, were still different from what would have been expected based on observed species proportions. This result indicated that the diversity effects on these variables were due to one or more species having different quality in mixtures than in pure stands. Analyses of nutritional quality in species-separated samples from the first and last harvests in the second year in the three-cut system showed that the grass species had higher concentrations of CP, ADF and NDF, and lower concentrations of WSC, NFC and NE_L, when grown in mixtures compared with pure stands (Table 4). In the last harvest, the CP concentration in the grasses was 40–50% higher in mixtures than in pure stands. ADF and NDF concentrations were 15–24 and 10–18% higher, and NE_L concentrations 8–10% lower, when the grasses were grown in mixtures as compared to pure stands. In the last harvest, the two grass species differed in that the fibre and NE_L concentrations were not significantly affected in perennial ryegrass while they were in tall fescue. The concentrations of easily digestible carbohydrates (WSC, NFC) were much lower in mixtures than in pure stands for both grass species. At the first harvest, the concentration of WSC was 32–41% lower and at the last harvest it was 69–76% lower in mixtures than in pure stands. In red clover, the CP concentration was not affected by stand type at all. For the carbohydrate fractions, there was an opposite tendency to that seen in grasses, and in the last harvest, the WSC concentration was significantly higher in mixtures than in pure stands. The results did not appear to be influenced by reproductive development, as tall fescue did not produce flowering stems in the last harvest, while red clover produced a lot of flowering stems, and perennial ryegrass a limited amount (data not shown). Sorted samples of white clover were not analysed due to limited white clover biomass in the samples from these harvests.

Species diversity effects on annual yields of crude protein and energy

Due to the strong positive and transgressive diversity effect on DM yield (Table 2; Ergon *et al.*, 2016), there were also significant positive and transgressive diversity effects on annual yields of nutritional components in both cutting systems and both years. The predicted annual NE_L yield of a centroid mixture was 52–72% higher than in the average pure stand (Table 2 and Supplementary File Table S3 of Supporting Information). The annual yield of CP is of particular interest, as it provides information on the N status of the swards. The predicted annual CP yield of a centroid mixture was 60–83% higher than in the average pure stand and 7–27% higher than in the red clover pure stand, which was the species with the highest CP yield. When we estimated the effect of varying the proportion of one species in the seed mixture from 0·1 to 0·7 while keeping the seed weight ratio between the three other species constant at 1:1:1 (Figure 1a), the maximum accumulated NE_L yield over the 2 years was estimated when the red clover proportion was 0·1 (both cutting systems). Together with the white clover, this corresponds to a total legume proportion of 0·4. When we manipulated the white clover proportion, the maximum accumulated yield was obtained at a proportion of 0·1 in the three-cut system and 0·4 in the five-cut system, corresponding to a total legume proportion of 0·4 and 0·6. For perennial ryegrass, the optimal proportions were 0·5 and 0·3 in the three- and five-cut systems, respectively, while for tall fescue it was 0·3 and 0·4. This corresponds to total legume proportions of 0·33–0·43. When we estimated the effect of varying the species seed weight ratios in all combinations, but keeping the minimum proportion of each species at 0·1, we found the maximum

Table 3 Measured concentrations of nutritional components (g kg^{-1} DM or MJ kg^{-1} DM) compared with expected (Exp.) concentrations, based on concentrations in pure stands and either sown species proportions, or observed species proportions. Species proportions were visually observed for each plot before each harvest and an annual average weighted for DM yield was calculated. Values are averages across all mixture types and replicates (n = 10).

Cutting system	Year		Nutritional variable					
			CP	NDF	ADF	WSC	NFC	NE$_L$
Three-cut	Year 1	Measured	156	468	322	167	290	5·67
		Exp. sown	155	444	298*	196*	309*	5·86*
		Exp. observed	149	440	298*	207*	317*	5·88*
	Year 2	Measured	145	470	318	183	294	5·71
		Exp. sown	134	464	297*	220*	310*	5·85*
		Exp. observed	141	441*	304*	218*	318*	5·84*
Five-cut	Year 1	Measured	160	471	310	159	290	5·80
		Exp. sown	164	433*	289*	193*	316*	6·01*
		Exp. observed	146	424*	277*	197*	304*	5·73
	Year 2	Measured	172	420	267	194	321	6·13
		Exp. sown	166	401	261*	210	332*	6·18
		Exp. observed	163	407	258*	218*	335*	6·20*

NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water-soluble carbohydrates; NFC, non-fibre carbohydrates; NE$_L$, net energy for lactation.
*, significantly different from the measured concentration (LSD, P < 0·05).

Table 4 Concentration of nutritive components (g kg^{-1} DM or MJ kg^{-1} DM) in species grown in pure and in mixed stands at the first and third harvests in the second year of the three-cut system. Significance levels are given when the quality of a species grown in mixture was different from the same species grown in pure stand.

Species†	Harvest	Species composition	N‡	Nutritional variable					
				CP	NDF	ADF	WSC	NFC	NE$_L$
Lp	First	Pure stand	2	96	458	241	349	366	6·3
	First	Mixture	10	114	524*	298*	236*	293*	5·8*
	Third	Pure stand	2	113	585	372	139	256	5·5
	Third	Mixture	8	157*	583	388	33*	239*	5·4
Fa	First	Pure stand	2	82	520	284	314	318	5·9
	First	Mixture	10	111	573*	328*	186*	249*	5·4*
	Third	Pure stand	2	78	530	318	249	307	5·8
	Third	Mixture	9	120*	625**	390**	78**	217*	5·2*
Tp	First	Pure stand	2	234	302	237	141	374	6·4
	First	Mixture	7	232	289	221	162	386	6·5
	Third	Pure stand	1	159	457	407	86	284	5·3
	Third	Mixture	10	158	406	355	161**	317	5·6

Lp, *L. perenne;* Fa, *F. arundinacea*; Tp, *Trifolium pratense*; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water-soluble carbohydrates; NFC, non-fibre carbohydrates; NE$_L$, net energy for lactation; DE, digestible energy; ME, metabolizable energy.
***, P < 0·0001; **, 0·0001 < P < 0·005; *, 0·005 < P < 0·05.
†White clover was not analysed due to limited biomass in the samples.
‡Samples from both replicate blocks and five mixtures with different relative sown species proportions were included, unless when there was not enough biomass of the species in the sample.

accumulated NE$_L$ yield in the three-cut system was obtained at proportions of 0·1 (red clover), 0·1 (white clover), 0·4 (tall fescue) and 0·4 (perennial ryegrass), while the maximum accumulated NE$_L$ yield in the five-cut system was obtained at proportions of 0·1 (red clover), 0·3 (white clover), 0·3 (tall fescue) and 0·3 (perennial ryegrass) (Figure 1b).

Intra-annual stability of nutritional quality

Mixtures had lower intra-annual variability than pure stands for NDF, ADF, WSC, NFC and all three energy concentrations in both the two-first years, and for CP in the second year only ($\chi^2_{,6\mathrm{df}}$ = 29·4–62·3, P < 0·0001; Figure 2).

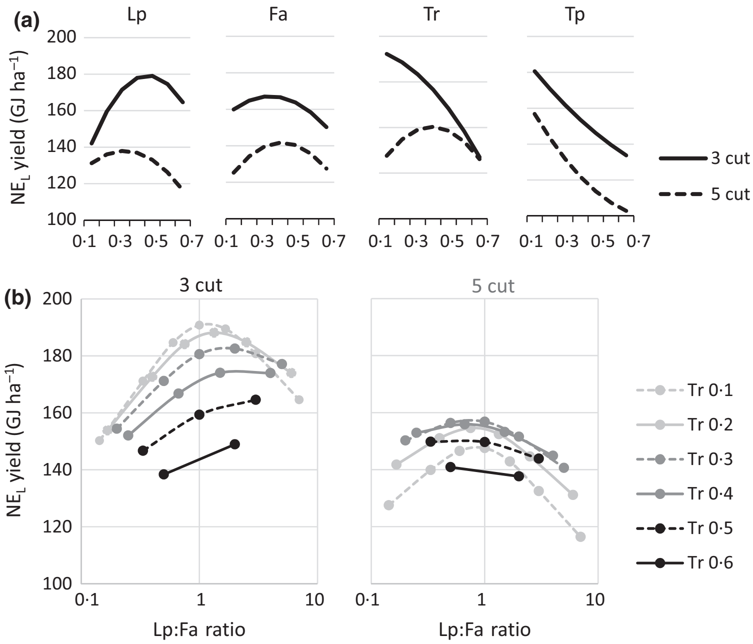

Figure 1 Estimation of total NE$_L$ yield accumulated over the two-first years in a three-cut and a five-cut system as a response to sown species composition (proportions of seed weight), using estimated species identity and species interaction coefficients for each species pair, cutting system and year. Lp, *Lolium perenne*; Fa, *Festuca arundinacea*; Tr, *Trifolium repens*; Tp, *T. pratense*. a) The proportion of the indicated species was varied from 0·1 to 0·7, keeping the ratios between the three other species constant at 1:1:1. b) The proportion of Tp was kept at 0·1 and the proportion of Tr was varied from 0·1 to 0·6. The ratio between Lp and Fa (*x*-axis, logarithmic scale) was varied within each level of Tr or Tp proportion.

Discussion

Differences between the species in CP, NDF, ADF and WSC content agreed with expectations. We found that there were significant species diversity effects on the nutritional composition of herbage from mixtures in the two-first years of the three-cut system and in the first year of the five-cut system. Mixtures had lower WSC and NFC concentrations, higher ADF concentrations and slightly lower NE$_L$ concentration than expected from the nutritional composition of pure stands. The diversity effects on WSC and ADF could not be explained by a change in species composition after sowing, indicating that changes in the nutritional composition in one or more species were involved. This was supported by the analysis of nutritional composition of single species sorted from the mixtures which were sampled from the first and last harvests in the three-cut system the second year. These analyses showed that the grass species had higher concentrations of fibre and CP and lower concentrations of easily digestible carbohydrates and NE$_L$ in mixtures than in pure stands. This may be an effect of higher availability of N, increased standing biomass, higher competition for light and lower leaf-to-stem ratios, in the mixtures as compared to the average pure stands (see Ergon *et al.*, 2016). Similarly, Gierus *et al.* (2012) found that perennial ryegrass had a higher NDF concentration and a lower NE$_L$ concentration when grown together with lucerne than when grown in pure stand, and this coincided with a higher DM yield of the mixture. A reduction in concentrations of WSC, accompanied by an increase in concentrations of CP, was observed in perennial ryegrasses in mixtures with white clover by Evans *et al.* (1996). The difference between mixtures and pure stands was particularly pronounced during late summer, when clover growth was strongest. Evans *et al.* proposed that when growth demand for fixed carbon exceeds supply, such as when plants are shaded, WSC levels are reduced. Consequently, high summer yield of legumes in mixtures may result in a reduction in WSC content of the companion grass due to shading. No fertilizer was applied in the experiment reported by Evans *et al.*, and differences in N availability and growth may therefore also have played a role. The lack of significant diversity effects on nutritional quality in the second year of the five-cut system in our experiment may be related to the lower DM yield and less shading there. In addition, unlike in 2012, the harvests in the five-cut system in 2011 were very unequal, with the second and fourth average mixture harvest comprising 32 and 37% of the annual harvest respectively (data not shown). This may have caused a stronger diversity effect on nutritional quality in the five-cut system in 2011 than if the harvests had been more evenly spaced.

Mixtures of grassland species have a higher light interception relative to pure stands (Spehn *et al.*, 2005) and grass species have longer leaves and shoots and invest more in supporting tissues and specific leaf area when grown in mixtures, particularly if legumes are present, indicating a role of N nutrition (Gubsch *et al.*, 2011). It is also known that N fertilization

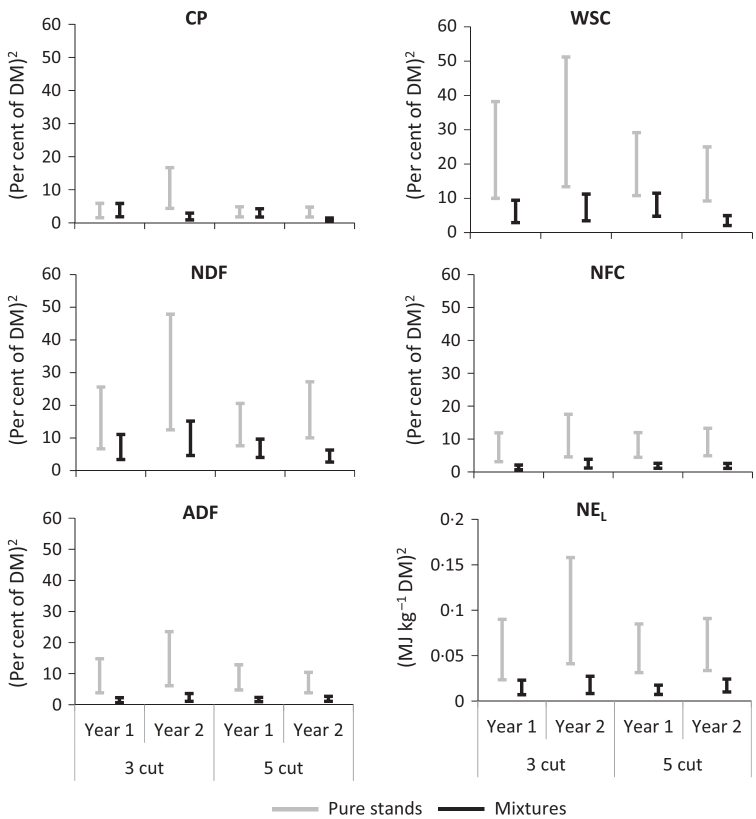

Figure 2 Confidence intervals of the mean (95%) for the intra-annual variability of nutritional components in the harvested pure stands of *Lolium perenne*, *Festuca pratensis*, *Trifolium repens* and *T. pratense* (grey) or mixtures of these species (black) in the two-first harvesting years (Y1 and Y2) in a three-cut system and a five-cut system. The intra-annual variability was estimated as variance components according to Model 2. CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water-soluble carbohydrates; NFC, non-fibre carbohydrates, NE_L, net energy for lactation.

changes the chemical composition of plants; CP concentration increases and WSC concentration decreases while the effects on structural carbohydrates vary (Peyraud and Astigarraga, 1998; Hoekstra *et al.*, 2007). To what extent N fertilization has an effect on structural carbohydrates may depend on whether the plants are in a vegetative or reproductive growth stage. Calvière and Duru (1999) found an increase in the stem proportion of spring growth grass herbage with increasing N and P status. We found, however, that even in the absence of reproductive stems in the third harvest, tall fescue had significantly more structural carbohydrates when grown in mixtures than in pure stands with low N supply. We also observed that tall fescue grew longer leaves than perennial ryegrass in mixtures in the three-cut system, which may have been associated with a higher proportion of ADF-rich supporting tissues in tall fescue. The negative effect of species diversity on nutritional quality in the grass component was not seen in red clover; in fact, species diversity tended to have the opposite effect. Unlike the other species, red clover may have experienced more competition for light in the pure stands than in mixtures, as the seeding rate used is high for legumes, and individual plants in pure stands can become very large. Higher levels of competition in pure stands may have caused a higher stem-to-leaf ratio and lower WSC concentration than in red clover plants grown in mixtures.

Sturludóttir *et al.* (2014) studied mixtures of timothy, smooth meadow grass, white and red clover in Nordic and Canadian conditions at low N fertilization levels (40–80 kg ha^{-1}) and two cuts per year. They analysed results across six sites and found strong diversity effects on DM yield over 3 years, but the diversity effect on nutritional quality was marginal. They did observe species interactions on nutrient variables, but these were both positive and negative and tended to cancel each other out. In contrast, we observed a net effect on several nutrient variables, which may be due to specific climatic or management conditions. Moreover, different grass species may vary in their responses to competition for light, their plasticity in stem-to-leaf ratios and the differences in chemical composition between stems and leaves.

The diversity effect on nutritional quality observed in this study is likely to be associated with the low level of N fertilization. Pure grass swards would normally receive higher levels of N fertilization than applied here, and this may be expected to have a similar effect on nutritional quality as cultivation in mixtures with legumes. However, although nutritional quality (in terms of the concentration of easily digestible carbohydrates) was somewhat lower in mixtures than in pure stands, there was still a very strong positive effect of diversity on NE_L and CP yield. This is mainly due to the strong positive diversity effect on DM yield (Ergon *et al.*, 2016). In addition, grasses absorb soil N efficiently, which has been shown to increase the symbiotic N acquisition of legumes (Nyfeler *et al.*, 2011). Estimations indicated that optimum seed weight proportions for accumulated DM yield over 3 years in four-species seed mixtures of red clover, white clover, perennial ryegrass and tall fescue was 0·1, 0·2, 0·4, 0·3 (three-cut system) and 0·1, 0·3, 0·3, 0·3 (five-cut system; Ergon *et al.*, 2016). Due to the effect of species diversity on NE_L concentration, the optimum proportions regarding accumulated NE_L yield over the two-first years, studied here, shifted in favour of less white clover in the three-cut system (0·1, 0·1, 0·4, 0·4). This likely reflects the stronger species diversity effect on nutritional quality in the three-cut system than in the five-cut system.

High intra-annual nutritional stability is often desired, especially in the context of practical farming. We found that the intra-annual nutritional stability was significantly higher in the average mixture than in the average pure stand in both cutting systems. This may partly be due to the fact that the four species have different seasonal patterns of stem formation and reproductive development. Previous research has shown that a combination of species in a mixture can result in a spread of herbage maturity through the year. For example, results from a mixture of three species (lucerne plus two grasses) grown in Argentina showed that although all three species were most mature in the summer, the grasses increased in maturity index during the spring, and lucerne extended its maturity through the autumn (Machado *et al.*, 2007).

In conclusion, in our three-cut system with low N fertilization, we found a significant species diversity effect on the chemical composition of forage harvested from mixed swards, leading to a 10% increase in ADF concentration, a 20–22% reduction in WSC concentration and a 4% reduction in NE_L concentration averaged across the two-first years. This diversity effect was at least partly due to reduced concentrations of WSC and increased concentrations of ADF in the grass component of mixtures compared with pure grass stands. An effect of species diversity in the five-cut system was only found in the first year. We have also demonstrated that species diversity strongly reduces the intra-annual variability in nutritional quality at two different cutting frequencies.

Acknowledgments

This work has received funding from the European Community's Seventh Framework Programme (FP7/2007–2013) under the grant agreement number FP7-244 983 (MULTISWARD). Arne Oddvar Skjelvåg has given valuable comments to the manuscript. Øyvind Jørgensen and other technicians at Norwegian University of Life Sciences and Norwegian Institute of Bioeconomy Research have provided technical assistance.

References

Brink G.E., Sanderson M.A. and Casler M.D. (2015) Grass and legume effects on nutritive value of complex forage mixtures. *Crop Science*, **55**, 1329–1337.

Calvière I. and Duru M. (1999) The effect of N and P fertilizer application and botanical composition on the leaf/stem ratio patterns in spring in Pyrenean meadows. *Grass and Forage Science*, **54**, 255–266.

Deak A., Hall M.H., Sanderson M.A. and Archibald D.D. (2007) Production and nutritive value of grazed simple and complex forage mixtures. *Agronomy Journal*, **99**, 814–821.

Duru M. (2008) Improvement of time-driven models of lamina cocksfoot digestibility by a process-based model to take account of plant N nutrition and defoliation. *Journal of Agronomy and Crop Science*, **194**, 401–412.

Ergon Å., Kirwan L., Bleken M.A., Skjelvåg A.O., Collins R. and Rognli O.A. (2016) Species interactions in grassland mixtures under low nitrogen fertilization and two cutting frequencies I: dry matter yield and stability and dynamics of species composition. *Grass and Forage Science*, **71**, 667–682.

Evans D.R., Humphreys M.O. and Williams T.R. (1996) Forage yield and quality interactions between white clover and contrasting ryegrass varieties in grazed swards. *Journal of Agricultural Science*, **126**, 295–299.

Gardarin A., Garnier E., Carrère P., Cruz P., Andueza D., Bonis A., Colace M.-P., Dumont B., Duru M., Farruggia A., Gaucherand S., Grigulis K., Lavorel S., Louault F., Loucougaray G., Mesléard F., Yavercovsky N. and Kazakou E. (2014) Plant trait-digestibility relationships across management and climate gradients in permanent grasslands. *Journal of Applied Ecology*, **51**, 1207–1217.

Gierus M., Kleen J. and Taube F. (2012) Forage legume species determine the nutritional quality of binary mixtures with perennial ryegrass in the first production year. *Animal Feed Science and Technology*, **172**, 150–161.

Gubsch M., Buchmann N., Schmid B., Schulze E.D., Lipowsky A. and Roscher C. (2011) Differential effects of plant diversity on functional trait variation of grass species. *Annals of Botany*, **107**, 157–169.

Hatfield R.D., Jung H.-J.D., Broderick G. and Jenkins T.C. (2007) Nutritional chemistry of forages. In: Barnes R.F., Nelson C.J., Moore K.J. and Collins M. (eds) *Forages: the science of grassland agriculture*, Vol. **II**, pp. 467–485. Ames, IA, USA: Blackwell Publishing.

Hoekstra N.J., Schulte R.P.O., Struik P.C. and Latinga E.A. (2007) Pathways to improving the N efficiency of grazing bovines. *European Journal of Agronomy*, **26**, 363–374.

Kirwan L., Lüscher A., Sebastià M.-T., Finn J.A., Collins R.P., Porqueddu C., Helgadóttir Á., Baadshaug O.H., Brophy C., Coran C., Dalmannsdóttir S., Delgado I., Elgersma A., Fothergill M., Frankow-Lindberg B.E., Golinski P., Grieu P., Gustavsson A., Höglind M., Huguenin-Elie O., Iliadis C., Jørgensen M., Kadziuliene Z., Karyotis C., Lunnan T., Malengier M., Maltoni S., Meyer V., Nyfeler D., Nykänen-Kurki P., Parente J., Smit H.J., Thumm U. and Connolly J. (2007) Evenness drives consistent diversity effects in intensive grassland systems across 28 European sites. *Journal of Ecology*, **95**, 530–539.

Kirwan L., Connolly J., Finn J.A., Brophy C., Lüscher A., Nyfeler D. and Sebastià M.-T. (2009) Diversity-interaction modeling: estimating contributions of species identities and interactions to ecosystem function. *Ecology*, **90**, 2032–2038.

Lüscher A., Mueller-Harvey L., Soussana J.F., Rees R.M. and Peyraud J.L. (2014) Potential of legume-based grassland-livestock systems in Europe: a review. *Grass and Forage Science*, **69**, 206–228.

Machado C.F., Morris S.T., Hodgson J., Matthew C. and Auza N. (2007) Seasonal variation in the quality of a lucerne-based pasture and its relationship with morphological and maturity estimates. *Australian Journal of Experimental Agriculture*, **47**, 575–582.

Moore K.J. and Jung H.G. (2001) Lignin and fibre digestion. *Journal of Range Management*, **54**, 420–430.

Nyfeler D., Huguenin-Eile O., Suter M., Frossard E. and Lüscher A. (2011) Grass-legume mixtures can yield more nitrogen than legume pure stands due to mutual stimulation of nitrogen uptake from symbiotic and non-symbiotic sources. *Agriculture, Ecosystems and Environment*, **140**, 155–163.

Peyraud J.L. and Astigarraga L. (1998) Review of the effect of nitrogen fertilization on the chemical composition, intake, digestion and nutritive value of fresh herbage: consequences on animal nutrition and N balance. *Animal Feed Science and Technology*, **72**, 235–259.

Picasso V.D., Brummer E.C., Liebman M., Dixon P.M. and Wilsey B.J. (2008) Crop species diversity affects productivity and weed suppression in perennial polycultures under two management strategies. *Crop Science*, **48**, 331–342.

Sanderson M.A. (2010) Stability of production and plant species diversity in managed grasslands: a retrospective study. *Basic and Applied Ecology*, **11**, 216–224.

Sleugh B., Moore K.K., George J.R. and Brummer E.C. (2000) Binary legume-grass mixtures improve forage yield, quality, and seasonal distribution. *Agronomy Journal*, **92**, 24–29.

Spehn E.M., Hector A., Joshi J., Sherer-Lorenzen M., Schmid B., Bazeley-White E., Beierkuhnlein C., Caldeira M.C., Diemer M., Dimitrakopoulus P.G., Finn J.A., Freitas H., Giller P.S., Good J., Harris R., Högberg P., Huss-Danell K., Jumpponen A., Korcheva J., Leadley P.W., Loreau M., Minns A., Mulder C.P.H., O'Donovan G., Otway S.J., Palmborg C., Pereira S., Pfisterer A.B., Prinz A., Read D.J., Schulze D.-J., Siamantziouras A.-S.D., Terry A.C., Trumbis A.Y., Woodward F.I., Yachi S. and Lawton J.H. (2005) Ecosystem effects of biodiversity manipulations in European grasslands. *Ecological Monographs*, **75**, 37–63.

Sturludóttir E., Brophy C., Bélanger G., Gustavsson A.-M., Jørgensen M., Lunnan T. and Helgadóttir Á. (2014) Benefits of mixing grasses and legumes for herbage yield and nutritive value in Northern Europe and Canada. *Grass and Forage Science*, **69**, 229–240.

Tracy B.F., Renne I.J., Gerrish J. and Sanderson M.A. (2004) Effects of plant diversity on invasion of weed species in experimental pasture communities. *Basic and Applied Ecology*, **5**, 543–550.

Van Soest P.J. and Fox D.G. (1992) Discounts for net energy and protein - 5th Revision. Proceedings of the Cornell nutrition conference, 1992, pp. 40–48.

Weiss W.P. (1993) Predicting the energy values of feeds. *Journal of Dairy Science*, **76**, 1802–1811.

Weiss W.P. (1995) *Theoretical models for estimating available energy concentrations in ruminant feeds*. Conseil des Productions Animales du Quebec (C.P.A.Q., Inc), p. 96.

Weiss W.P., Conrad H.R. and Pierre N.S. (1992) A theoretically-based model for predicting total digestible nutrient values of forages and concentrates. *Animal Feed Science and Technology*, **39**, 95–110.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Calibration and validation statistics for the ability of near-infrared spectroscopy to predict the nutritive attributes of the validation samples. CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; NFC, non-fibre carbohydrates, WSC, water-soluble carbohydrates, NE_L, net energy for lactation; DE, digestible energy; ME, metabolizable energy.
Table S2. A) Weighted annual concentrations (g $(kg\ DM)^{-1}$ or MJ $(kg\ DM)^{-1}$) and B) annual yields (t ha^{-1} or GJ ha^{-1}) of quality components measured in different sown stand types in the two-first years after the sowing year. Lp, *Lolium perenne*; Fa, *Festuca arundinacea*; Tr, *Trifolium repens*; Tp, *T. pratense*; _p, pure stand; C, centroid (25% seed weight of each species sown); _d, dominated (67% of the indicated species, 11% of each of the three other species sown). CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; WSC, water-soluble

carbohydrates; NFC, non-fiber carbohydrates; NE_L, net energy for lactation. The averages of two replicate blocks are given.

Table S3. Parameter estimates for species identity coefficients β (Lp, *Lolium perenne*; Fa, *Festuca arundinacea;* Tr, *Trifolium repens*; Tp, *T. pratense*) and the species diversity coefficient δ, on the yield of net energy for lactation (NE_L, GJ ha^{-1}) and crude protein (CP, t ha^{-1}) in mixtures of the four species. The species identity coefficient equals the variable estimates in pure stands, while the species diversity coefficient equals the variable estimate in a centroid mixture minus the average of pure stands. All estimates were significant at $P < 0{\cdot}0002$.