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Reducing nitrogen use and improving feed quality of grassland: Combining red clover with grasses with targeted traits

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Dear Editor,

Please find attached our manuscript entitled *"Reducing nitrogen use and improving feed quality of grassland: Combining red clover with grasses with targeted traits"* submitted for consideration for publication in *Field Crops Research*.

The focus of this work relates to the impact of grasses with novel traits intercropped with red clover within short-medium term leys in temperate cropping systems. Grasslands cover an estimated quarter of the world surface and, together with livestock, account for up to half of the technical mitigation-potential of agriculture through management options that sustainably intensify livestock production. Innovative varieties for short-medium term leys may provide a way to increase the productivity of leys, enhance resilience of pasture systems and reduce fertiliser inputs. Within this manuscript we examine sward performance in terms of forage quality and quantity, nitrogen transfer from clover to neighbouring grasses, and grass rooting depth along with soil N and C pools at depth. Additionally, we apply two measures of nitrogen use efficiency as a metric for agronomic performance of the grass varieties examined. Highlighting areas for optimising the performance of short-medium term leys. We believe this experimental research into grasses with specific traits and the agronomic metrics reported conducted is well suited to the scope of *Field Crops Research*.

We trust that this manuscript will be of interest to a broad range of readers, including land managers, academics and policy makers, particularly those interested in optimising short-medium term leys for improved resilience and reducing the impact of livestock in grass fed systems on the wider environment. We look forward to hearing from you.

Sincerely yours,

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Manuscript File

Reducing nitrogen use and improving feed quality of grassland: Combining red clover with grasses with targeted traits

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Highlights

- Herbage yields were greater when red clover was included within the sward
- Inclusion of red clover with grass increased sward N and metabolisable energy content
- Including red clover in grass swards replaced the need for N fertiliser
- *Festulolium* gave no yield advantage over a ryegrass hybrid under drought conditions
- The greater root mass of *festulolium* did not affect soil C and N content at depth

Abstract

To increase ruminant production efficiency, the environmental impact of growing forage must be reduced. Here we examined the role of red clover (cv. AberClaret) in minimising nitrogen (N) requirements, alongside two novel grass varieties, (1) a *festulolium* (cv. AberNiche), developed for drought tolerance, with potential for deep-rooting, and (2) a ryegrass hybrid (cv. AberEcho), developed for high-sugar content, which may enhance ruminant N-uptake *in-vivo*. Field trials were conducted at two sites growing *festulolium* and ryegrass ± red clover (at 29% of the seed mix weight), at a range of N fertilisation rates (0 – 600 kg N ha^{-1}), for 2 years (six harvests). We assessed sward performance (N offtake, herbage and silage quality, grass N use efficiency), rooting depth and N transfer from clover to grasses using ^{15}N natural abundance. Across both sites and years, dry matter and herbage-N content were overall greater from the swards that included clover. Yields from *festulolium* were not greater than from ryegrass under the drought conditions experienced, despite its greater root mass. Agronomic efficiency of fertiliser N was similar between grasses (19 - 22 %), however the *festulolium* more effectively used endogenous soil N than the ryegrass. There was no difference in soil N and C profiles between the two grasses. Inclusion of clover in the sward positively affected forage quality (crude protein, metabolisable energy), but reduced sugar and fibre (NDF) content. Among the grass types, metabolisable energy was greater and NDF content less for ryegrass than for *festulolium*. The effect of clover within the sward carried through to the ensiled herbage with increased N and reduced sugar and fibre in the silage from the clover mixed swards, relative to the single species grass swards. A strong reliance on biological N fixation (80 – 94%) for clover was observed, however, N transfer from clover to the neighbouring grass was not evident from the $\delta^{15}N$ signatures. Inclusion of grass varieties that can deep-root or provide high-sugar content had no impact on yield, but herbage quality was relatively better for ryegrass. The capacity for *festulolium* to (i) deep-root and enhance grassland resilience under a prolonged drought, and (ii) promote deep soil C storage was not observed in this study. We conclude that red clover is a viable fertiliser-N replacement strategy in short-term leys, and that grass varieties with improved herbage

quality may provide a better option for optimising sward performance than drought tolerant grass varieties.

Keywords

Fertiliser response, livestock production, NUE, partial factor productivity, plant trait

1. Introduction

The efficiency of nitrogen (N) use in ruminant production systems remains low (Leach et al., 2012; de Klein et al., 2017; Carswell et al., 2019a). However, there are opportunities for improving it, such as in feed and forage production (Misselbrook et al., 2013; Eisler et al., 2014) and the management of excreta (Ma et al., 2010); this study focuses on the former. Grasslands account for 3.2 x 10^9 hectares of the worldwide agricultural area (FAO, 2018). Under intensive grassland systems, pasture performance can be enhanced through N fertilisation, particularly for single-species grass swards. When legumes such as clover, which source N via biological N fixation (BNF), are incorporated within the sward, grasslands can be highly productive (Reid et al., 1970; Burchill et al., 2014; Enriquez-Hidalgo et al., 2016). However, the yield response of mixed grass and clover swards can be suppressed by N fertilisation (Reid et al., 1970; Enriquez-Hidalgo et al., 2016).

Red clover can supply a large amount of N when included within grassland swards (ca. 150 - 250 kg^{-1} N ha^{-1} y^{-1}, AHDB 2016; Marshall et al., 2017). In addition to sourcing N from BNF for its own use, red clover can also become a N donor and directly transfer BNF sourced-N to neighbouring grasses (Pirhofer-Walzl et al., 2012), negating the need for additional N fertilisation. Further benefits of incorporating red clover within grassland swards include the provision of the enzyme polyphenol oxidase, which, with its lipid-protecting role, can lead to increased levels of polyunsaturated fatty acids in milk and meat from ruminants (van Ranst et al., 2011). Polyphenol oxidase has also been linked to reduced proteolysis during the ensiling process, which is important for forage preservation (Jones et al., 1995). Although the benefits of red clover within swards are well established, difficulties in long-term persistency within swards (> 3 y) can occur (Eriksen et al., 2012; Marshall et al., 2017), therefore its inclusion may be limited to short-medium term leys within crop rotations.

When sowing a new ley, there is the opportunity to choose a sward including species with specific traits, such as the ability to deep-root, or produce high-sugar content within the herbage (Kell, 2011). *Festulolium* (e.g. *Lolium multiflorum* × *Festuca pratensis)* has been developed for both drought and cold tolerance (Ghesquière et al., 2010). The potential for *festulolium* to produce greater root mass and to deep-root has been associated with other benefits, such as increasing rainwater lag times to receiving water bodies (Macleod et al., 2013), and speculation that deep-rooting might be associated with increased carbon (C) sequestration or indeed increased turnover of C at depth (Marshall et al., 2016). In contrast to *festulolium*, high-sugar grasses have been bred to optimise *in-vivo* N use efficiency in ruminants. When energy supply is low within the rumen, microbes resort to amino acids for energy supply rather than assimilating them into microbial protein. This in turn leads to ammonia accumulating within the rumen, which is subsequently lost from the animal as urea-N (Miller et al., 2001). Merry et al. (2006) investigated the inclusion of red clover within a high-sugar grass silage and demonstrated that conversion of feed-N to microbial-N was increased when red clover silage was mixed with high-sugar grass silage, as was the efficiency of microbial protein synthesis, relative to red clover silage alone. Thus, grasses with high-sugar content, mixed with red clover, may enhance feed N efficiency and reduce ruminant N losses.

The objectives of this study were to test the hypotheses that: (1) including red clover in the sward would negate the need for N fertiliser, with BNF providing N to the clover and to neighbouring grasses; (2) inclusion of red clover would not detrimentally affect the forage (herbage or silage) quality; (3) a *festulolium* would produce greater yields under dry conditions relative to the hybrid ryegrass examined; (4) enhanced rooting and nutrient cycling at depth would be observed with the *festulolium* relative to the ryegrass hybrid, but that sufficient N supply would suppress deep-rooting; and (5) that plant uptake of applied N would be greater for the *festulolium* due to its greater rooting potential.

2. Materials and methods

2.1. Site description

The plot trials were conducted over two growing seasons (2017 and 2018) at two sites in the UK, see Carswell et al. (2019b) for full site descriptions. The first site was at Rothamsted Research – North Wyke (NW), in the southwest of England (50°46’39”N, 3°54’30”W, 128 m a.s.l.), with an average annual temperature 9.6 °C and annual precipitation of 1056 mm (40-year average for research station; Harrod and Hogan, 2008). The NW site was previously a permanent pasture. The second site was at Henfaes Research Station – Bangor University (HF), in North Wales (53°14’19”N, 4°01’09”W, 15 m a.s.l.), with an average annual temperature of 10.4 °C and annual precipitation of 830 mm (40-year average for Valley, Anglesey; Met Office, 2020). The previous two growing seasons at the HF site was temporary grass ley. The background soil characteristics, determined after ploughing and reseeding of new swards (in Autumn 2016), are presented in Table 1.

Table 1. Background soil properties. Values represent means ± standard error (*n* = 4 at Henfaes and n = 5 at North Wyke).

Soil property (0 – 10 cm depth)	North Wyke	Henfaes
Soil classification (FAO)	Gleyi-eutric Fluvisol	Eutric Cambisol
Textural classification	Clay loam	Sandy clay loam
pH (1:2.5; soil:water)	5.7 ± 0.2	6.5 ± 0.1
EC (1:2.5; soil:water; µS cm^{-1})	21.8 ± 1.3	27.5 ± 1.5
Bulk density (g cm^{-3})	1.01 ± 0.05	0.99 ± 0.01
Total soil C (g kg^{-1} DW)	29.3 ± 1.5	26.5 ± 1.0
Total soil N (g kg^{-1} DW)	3.29 ± 0.15	2.49 ± 0.07
Available* soil P (g kg^{-1} DW)	2.10 ± 0.26	2.11 ± 0.11
Soil C:N ratio	8.88 ± 0.07	10.6 ± 0.16
Dissolved organic C (as NPOC; mg kg^{-1} DW)	123 ± 6	95 ± 3
Dissolved organic N (mg kg^{-1} DW)	17 ± 1	23 ± 1
NH_4-N (mg kg^{-1} DW)	2.78 ± 0.60	1.15 ± 0.13
Total oxidised N (NO_3-N + NO_2-N; mg kg^{-1} DW)	2.81 ± 0.48	2.61 ± 0.30
Total mineralisable N (mg kg^{-1} DW)	43.6 ± 3.7	52.6 ± 2.7

EC = electrical conductivity; DW is dry weight equivalent; NPOC = non-purgeable organic C; *Available soil P = extractable with 0.5 M $C_2H_4O_2$.

2.2. Experimental design

The plot-scale experiment consisted of four swards at each site, including (1) a single species sward of *festulolium* (*Lolium multiflorum* × *Festuca pratensis*; cv. AberNiche; Humphreys et al., 2014), henceforth treatment "F", (2) a single species sward of a hybrid ryegrass (*Lolium perenne* × *Lolium multiflorum*; tetraploid, cv. AberEcho), treatment "R", (3) F with *Trifolium pratense* (cv. AberClaret), treatment "FC", and (4) R with *Trifolium pratense*, treatment "RC". Seeds were sown at a rate of 30 kg ha^{-1} for the single species swards and at 20 kg grass seed ha^{-1} with 8 kg clover seed ha^{-1} for the mixed swards. At NW, all treatments were replicated five times, with a total of twenty plots measuring 72 m^2 in a balanced incomplete block design, whereas at HF sward treatments were replicated four times, with a total of sixteen plots measuring 90 m^2 in a randomised complete block design. The single species plots, F and R, were further split into five subplots at NW and six subplots at HF, to allow for multiple N (as ammonium nitrate) fertiliser rates. Nitrogen fertiliser was applied by hand at the equivalent rates of 0, 75, 150, 300, and 450 kg N ha^{-1}, with an additional rate of 600 kg N ha^{-1} at HF, split over three applications (see supplementary information Table S1 for fertilisation dates). The plots containing clover, i.e. RC and FC, were split into two subplots with N rates of 0 and 50 kg N ha^{-1}, applied as a single dose in early Spring. Additional fertilisers were applied to ensure P, K, S and Mg were not limiting according to soil tests and crop requirements (Defra, 2010).

2.3. Herbage quantity and quality

The swards were managed as a three-cut silage system, although a fourth cut was conducted at NW in 2017 due to local conditions allowing an extended growing season (Table S1). At both sites, herbage was cut along a swathe of fixed width and measured length (7 m^2 harvestable area at HF, and 6 m^2 harvestable area at NW) to a residual height of 5 cm, to allow metrics to be expressed on a per ha basis. Cut herbage was immediately sampled following cutting, with two subsamples taken from each subplot (70 subplots at NW and 64 subplots at HF). The first subsample was divided into clover and grass samples upon which dry matter (DM) was determined after drying at 80 °C to a constant weight, and at NW the total N and ^{15}N content was measured using a Carlo Erba NA 2000 linked to a Sercon 20/22 isotope ratio mass spectrometer (Sercon, Crewe, UK; Carlo Erba, CE

Instruments, Wigan, UK). Total N was determined at HF using a TrueSpec® analyser (Leco Corp., St Joseph, MI). The second subsample was retained as a whole-sward sample to determine whole-sward quality parameters including crude protein (CP), sugar, neutral detergent fibre (NDF), and metabolisable energy (ME) content, with analyses conducted by Sciantec Analytical Laboratories, Stockbridge Technology Centre, York, UK and Trouw Nutrition GB, Blenheim House, Ashbourne, UK for HF and NW samples respectively.

2.4. Root sampling and analyses

The impacts of the different grass varieties on root development and nutrient cycling at depth was examined via the collection of intact soil cores, taken at the end of the second growing season at each site. Intact soil cores were taken from the F and R swards, from the 0 and 300 kg N ha^{-1} plots, to a depth of 1 m using a steel-corer with sheath (70 mm i.d.), adapted to fit a pneumatic breaker (Cobra percussion hammer corer; VanWalt Ltd., Haslemere, Surrey, UK). The pneumatic breaker was used to exert downward force to push the steel corer to 1 m depth. Cores were divided into the following seven sections immediately after sampling, 0-10, 10-20, 20-30, 30-40, 40-50, 50-75, 75-100 cm depth. All samples were stored at 4 °C prior to analyses. Sub-samples of the fresh soil containing no visible roots were extracted with 0.5 M K_2SO_4 at a 1:5 soil:extractant ratio (w/v) and the extractant analysed for total N, organic C (as non-purgeable organic C; using a Multi N/C 2100/2100 analyser; AnalytikJena AG, Jena, Germany), NH_4-N (according to Mulvaney, 1996) and total oxidised N (NO_3-N and NO_2-N; according to Miranda et al., 2001). The remainder of the soil-core sections were washed of soil and all visible roots removed above 1 mm in length and retained, root content of the sub-sample taken for soil analyses was assumed to be zero. Root dry weight was determined by drying at 80 °C until a constant weight was reached.

2.5. Simulated silage experiment

A simulated ensiling study was conducted at both sites in the second year (2018) to examine the influence of grass variety and clover intercropping on silage quality parameters. Ensiling of herbage, from both N rates of the FC and RC treatments and the 0 and 150 kg N ha^{-1} fertiliser rates of the F and R treatments, was conducted on the first cut (May/June) samples in 2018. Miniature silos were created in duplicate by placing approximately 100 g of herbage that had been wilted overnight into vacuum bags (polyethylene interior, polyamide exterior; 200 × 300 mm; The Vacuum Pouch Company, Bury, UK). After evacuating the silage bags of air, they were sealed and stored at 22 °C (± 0.5 °C) in the dark for 90 days (Johnson et al., 2005). After 90 days one sample (approximately 30 g DW) was freeze-dried prior to water-soluble carbohydrates [WSC; fructan, sucrose, glucose and fructose analysis, based on the method of Maharjan et al. (2017), adapted to separate co-eluting mannitol and fructose peaks; HPLC Agilent 1260 infinity with ELSD, Agilent, California, USA] analysis, and analyses of NDF and ash content (Clancy and Wilson, 1966). The second sample was opened, mixed and weighed. After weighing, a sample was taken for immediate pH measurement by placing a 10 g sample into 90 ml of deionised H_2O, the sample was homogenised using a stomacher for 2 min at 220 rev min^{-1} (Seward UK, Worthing, United Kingdom) and the pH of the supernatant measured using a pH probe (Jenway 3320, Cole Palmer, Staffordshire, UK). The remaining sample was dried at 80 °C until a constant weight was reached. Total N analysis was performed on the dried silage, as described above.

2.6. Data analyses

The Met Office monthly meteorological data (Met Office, 2020) at the HF site and the UK Environmental Change Network meteorology data (Rennie et al., 2017) at the NW site were used to describe the meteorological conditions experienced during the field trials. To assess the efficiency of N use by the single species grass swards two metrics of N use efficiency were applied to the herbage DM yield data, the agronomic efficiency of applied N (AE_N) and the partial factor productivity of applied N (PFP_N). The former reports the efficiency of crop recovery of applied N only, with the yield of the 0 N controls accounted for in the calculation, whereas the latter reports the efficiency of crop use of both applied and endogenous soil N (Dobermann, 2005). The proportion of N derived from atmosphere in red-clover shoots was calculated according to Unkovich et al. (2008), with two grasses (under zero N fertiliser) used as a reference for N derived from atmosphere from non-N fixing species (Zhang et al., 2020).

The treatment effects of sward composition and N rate on each of the measured parameters were assessed using linear mixed models (REML directive in Genstat v. 20.1, VSN International) to allow for the different designs at each site. All models had a nested random structure, Site/Block/Plot/Subplot/sample, apart from the silage quality parameters for which there was only a single sample per subplot. The fixed structure for N offtake (DM yield x N content of harvested material), DM yield, and herbage quality parameters was Grass * Clover/(N0 + N1) * Cut. This structure tests for differences due to grass variety (F or R), inclusion of clover, N rate when clover is included (N1), N rate when clover is excluded (N0) and cuts. The CP and sugar herbage quality data required a square root transformation to satisfy assumptions of equal variance and normality of residuals.

For the silage quality parameters (total N, pH, WSC, NDF and ash) the fixed structure was Grass * Clover/(N0 + N1) which tests for differences due to grass varieties, inclusion of clover, N rate when clover was included (N1) and N rate when clover was excluded (N0). The models fitted to AE_N and PFP_N used a crossed fixed structure to test the effects of grass type, N rate and year. Agronomic efficiency of N required a square root transformation with an offset and PFP_N required a log transformation to satisfy model assumptions. To examine differences between root mass, soil mineral N (SMN; ammonium + total oxidised N), soluble organic C (SOC) and soluble organic N (SON) at different rooting depths under the single species swards at 0 and 300 kg N ha^{-1} the fixed structure of the models had a crossed structure including grass variety, N rate and core depth. The root mass data contained multiple zeros (i.e. no roots present) and required a natural logarithmic data transformation and the SMN, SOC and SON data required a square root transformation to satisfy model assumptions.

General analysis of variance with Fishers LSD (Genstat v. 19.1, VSN International) was used to examine differences in the $\delta^{15}N$ signatures of the single species grass when grown with or without clover.

3. Results

3.1. Meteorological conditions

At NW, an extremely dry winter followed the autumn sowing of the treatments with precipitation levels of 21, 45 and 67% of the 40-year average (Harrod and Hogan, 2008) for December, January and February 2016/2017, respectively. This was accompanied by mild spring temperatures in February and March 2017, of 2.1 and 2.5°C above the 40-year average. Similar weather patterns were observed at HF, with precipitation at 26, 79 and 86% of the 40-year average (1971-2010 data for Valley, Anglesey; Met Office, 2020) for October, November and December. The following spring at HF was also mild with March, April and May temperatures at 1.6, 0.8 and 1.9 °C greater than the 40-year averages. The remainder of 2017 had temperatures typical of both sites. Annual precipitation in 2017 was below average at 918 mm for NW and above average at 871 mm for HF (respective long-term averages are 1056 and 830 mm; Harrod and Hogan, 2008; Met Office, 2020).

In contrast to 2017, 2018 was marked by a wet and cold spring with a total of 266 and 160 mm of precipitation falling in March and April together, at NW and HF respectively, and temperatures in February and March at both sites were below average, by 1.0 and 1.1 °C, at NW and 1.2 and 1.5 °C, at HF, respectively. The spring/summer of 2018 at NW was markedly warmer than average with temperatures of 2.0, 3.0 and 2.7 °C greater in May, June and, July respectively, and much reduced precipitation levels of 59, 6 and 60% in May, June and July respectively (Harrod and Hogan, 2008). The spring/summer of 2018 was also warmer at HF with temperatures in April, May, June and July 0.7, 1.0, 2.2 and 1.6 °C greater than the average, a substantially drier period was also observed, with precipitation during May, June, July and August at 61, 21, 93 and 55% of the 40-year average (Met Office, 2020). Consequently, the spring/summer of 2018, particularly May and June, can be considered a drought period at both field sites, allowing the opportunity to evaluate the *festulolium* under conditions relevant to its novel traits.

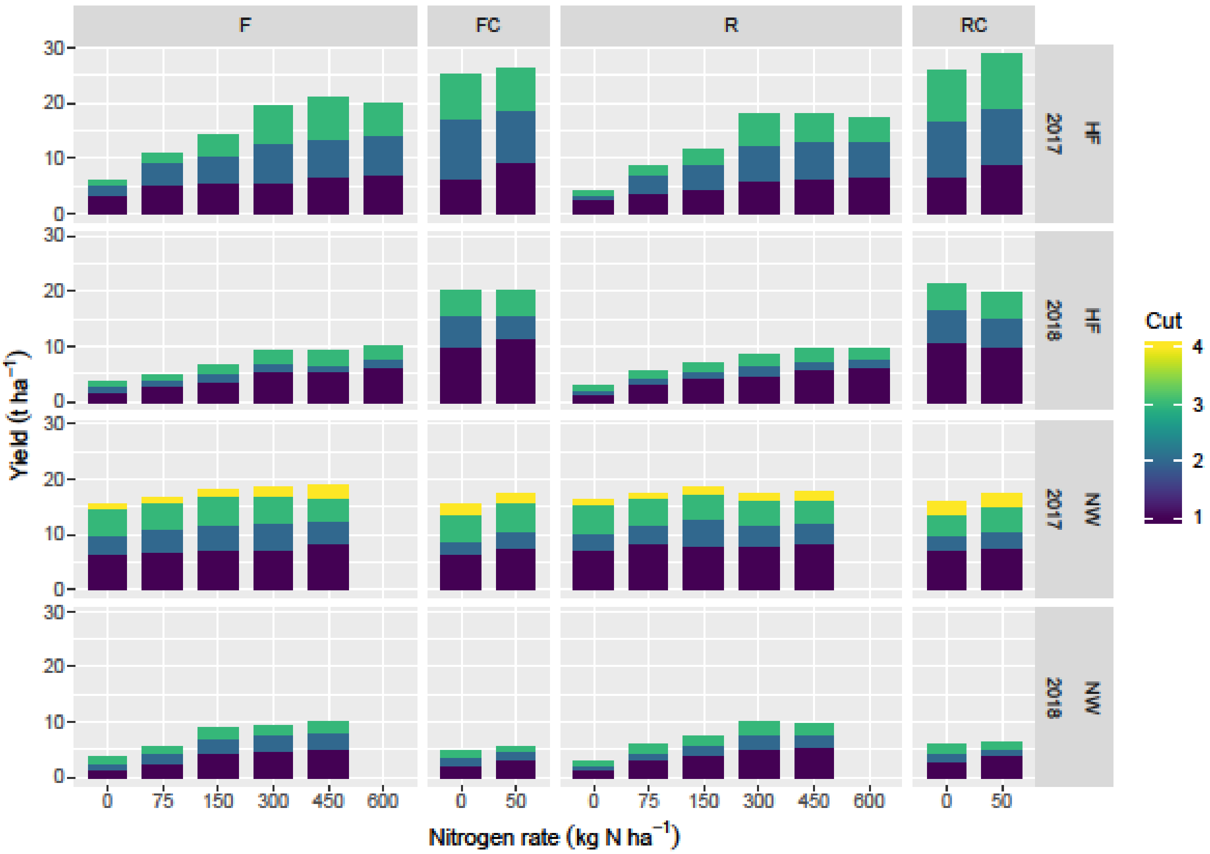
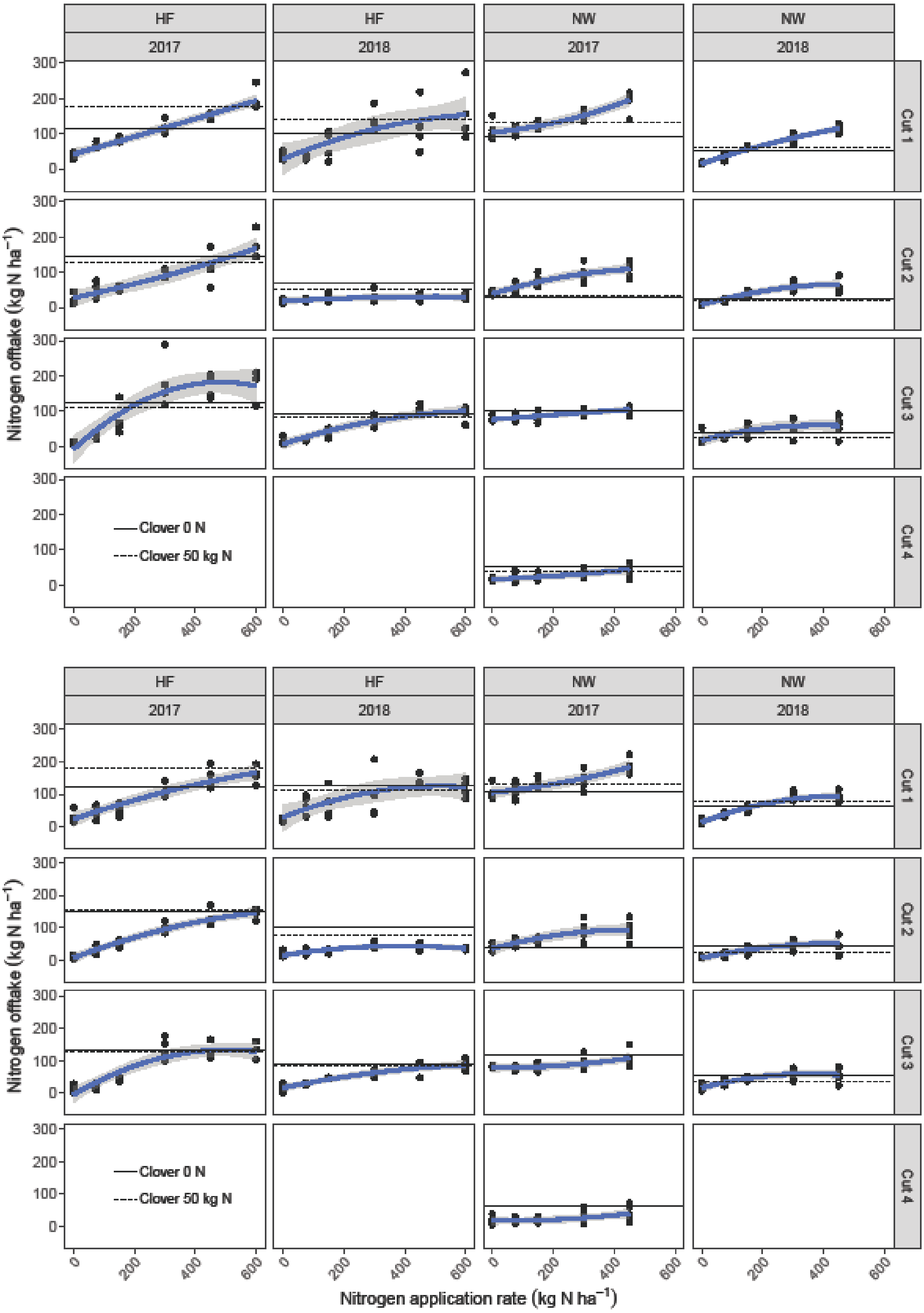


Figure 1. Dry matter yields with and without clover (C), for the *festulolium* (F) and the high-sugar ryegrass (R). Bars are mean values calculated on a cut-basis, where n = 4 for the Henfaes site (HF) and n = 5 for the North Wyke site (NW). See Table S2 for full dataset with standard errors.

3.2. Herbage yield and quality

Annual average DM yields across both sites and years were 14.2 ± 2.8 and 14.8 ± 2.9 t ha^{-1} for RC at 0 and 50 kg N ha^{-1} (± data refers to standard error of mean hereafter) respectively, and 14.1 ± 3.0 and 14.4 ± 3.0 t ha^{-1} for FC at 0 and 50 kg N ha^{-1}, respectively. The application of 50 kg N ha^{-1} in the spring did not significantly affect DM yields on the mixed swards (p = 0.581). Where clover was not present in the swards, DM yields were less (p < 0.001), with DM yields of 9.6 ± 1.2, 11.3 ± 1.4, 10.7 ± 1.3, and 12.5 ± 1.6 t ha^{-1} observed at N application rates of 150 and 300 kg N ha^{-1} for R and of 150 and 300 kg N ha^{-1} for F across both sites and years, respectively (Figure 1, see also Table S2).

As with DM yields, inclusion of clover in the sward had a significant effect on N offtake (p = 0.002), with greater N offtake achieved in the treatments with clover (Figure 2). Nitrogen application rate only affected N offtake in the single species sward ($p < 0.001$), with no effect observed in the mixed swards ($p = 0.566$). Greatest N offtakes were observed at the NW site in 2017, which can be linked to a high yielding first cut. Typically, lower N offtake was observed for all treatments in 2018 due to the summer drought limiting rhizosphere processes. However, exceptions to this occurred at the HF site for the single species R sward at lower N rates (0, 75 and 150 kg N ha^{-1}), with the 2018 dry weather only affecting these treatments by the third cut (Figure 2 and Table S2). Annual N offtake from the mixed swards receiving 0 kg N ha^{-1} was 297 ± 27 and 260 ± 27 kg N ha^{-1} for RC and FC respectively, which was equivalent to N offtake from the single species treatments at between 300 and 450 kg N ha^{-1} for R and between 150 and 300 kg N ha^{-1} for F. Thus, BNF was able to provide crop N yields equivalent to those of typical N application rates for intensive grasslands (150 - 300 kg N ha^{-1}).



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274 **Figure 2.** Nitrogen offtake at each cut for the clover mixed swards relative to the single species
275 swards. The upper panel presents the *festulolium* hybrid (F) with *Trifolium pratense* mix (FC), and the
276 lower panel presents the *Lolium perenne* × *Lolium multiflorum* hybrid (R) with *Trifolium pratense* mix
277 (RC). The solid and dashed lines indicate the mean herbage yield for the mixed swards at 0 and 50 kg

N ha^{-1} respectively, whereas, the data points present the individual replicate herbage yields from the single species swards at varying N application rates. The N response for the single species swards was best described by second order polynomial functions, shown in the trendline with standard error as the grey curved area.

Herbage quality parameters were measured on whole sward samples at all cuts (except cut 4 at NW in 2017; see Table S3 for mean values ± SEM) and examined across both sites and years together. The interquartile ranges for CP, NDF, sugar and ME were 103 and 166, 407 and 518, 71 and 168, and 10.5 and 11.4 g kg^{-1} DM respectively. Inclusion of clover within the swards had a significant positive effect on CP (p = 0.048) and ME (p = 0.003) content and a negative effect on sugar (p < 0.001) and NDF (p < 0.001) content. The same trade-off between CP *vs*. sugar was observed for N application rate on the single species swards, with positive trends observed for CP, and NDF up to the 300 kg N ha^{-1} application rate, and negative trends observed for sugar and ME (p < 0.001 for all). However, for the mixed swards, only sugar content was significantly affected by N application rate (p < 0.001) with greater sugar content from the 50 kg N ha^{-1} relative to the zero N treatment. The impact of weather and season was observed for all herbage quality parameters when differences between cuts were examined (p < 0.001 for CP, NDF, sugar and ME). The herbage quality for the two grass types (F and R) only differed for NDF and ME content (p < 0.001) with F having greater NDF and lesser ME relative to R. Nonetheless, the interaction between grass type and cut was significant for all parameters (p ≤ 0.023), with R having greater sugar and lesser NDF than F for the 2017-Cut 2 and 3, and 2018-Cut 2 for NDF only, again highlighting the importance of seasonal growth characteristics for optimal sward performance.

3.3. Nitrogen use efficiency of single species swards

The AE_N was statistically similar between both grass types (p = 0.224; see also Table 2). Perhaps unsurprisingly, only N application rate had a significant effect on AE_N (p < 0.001), reaching a maximum at the 150 kg N ha^{-1} application rate and a minimum at 600 kg N ha^{-1}. In contrast where both applied and endogenous N sources were accounted for under PFP_N the grass types demonstrated significant differences in their efficiency of N use (p < 0.001), with F achieving greater

PFP_N than R (Table 2). Year also had a significant effect on PFP_N ($p < 0.001$), with the low yields under the drought conditions experienced in 2018 making PFP_N 40% lower than that observed for 2017. As for AE_N, N application rate had a significant effect on PFP_N ($p < 0.001$), however the trend was for declining PFP_N with increasing N application rate.

Table 2. Nitrogen use efficiency metrics for the single species grass varieties.

		Agronomic efficiency of nitrogen (g DM g^{-1} N)	Partial factor productivity of nitrogen (g DM g^{-1} N)
Grass type	F	22.0 (3.7 - 44.2)	44.4 (40.9 - 48.1)
	R	19.3 (1.5 - 41.0)	40.3 (37.2 - 43.7)
Nitrogen rate (kg N ha^{-1})	75	27.8 (10.4 - 48.3)	99.3 (91.1 - 108.3)
	150	30.5 (12.7 - 51.4)	64.5 (59.1 - 70.3)
	300	21.8 (5.4 - 41.3)	37.7 (34.6 - 41.2)
	450	15.7 (0.4 - 34.1)	25.7 (23.6 - 28.0)
	600	8.9 (-5.6 - 26.8)	21. 8 (19.5 - 24.3)
Year	2017	21.0 (4.7 - 40.3)	54.5 (50.0 - 59.4)
	2018	20.3 (4.1 - 39.5)	32.8 (30.1 - 35.7)
Significance of fixed effects			
Grass type		0.22	< 0.001
Nitrogen rate		< 0.001	< 0.001
Year		0.86	< 0.001

DM = dry matter; F = *festulolium*; R = ryegrass (*Lolium perenne × Lolium multiflorum* hybrid); values are means with 95% confidence intervals in brackets

3.4. Silage quality

Silage quality parameters were measured on the first cut of 2018 at both sites (see Table S1 for cutting dates) from the single species swards, at the 0 and 150 kg N ha^{-1} application rates, and on the clover mixed swards, at the 0 and 50 kg N ha^{-1} application rates (Table 3). Across both sites, the presence of clover within the sward had a significant effect on all silage quality parameters examined ($p < 0.001$ for total N, ash, NDF, and WSC, and $p = 0.016$ for pH), with greater total N, ash and pH, and lower NDF and WSC relative to the single species swards. Within the swards containing clover, N application rate had a significant effect on ash ($p = 0.001$) and there was marginal evidence of an effect on NDF ($p = 0.061$), with greater ash and lower NDF for the 0 N treatment relative to the

50 kg N ha^{-1} treatment. Grass type was only observed to have an impact on ash content (p = 0.020) when examined for swards both with and without clover, and this effect was also seen in the interaction between grass type and N rate within the single species swards (p = 0.028), with F at 0 kg N ha^{-1} having a greater ash content than R at 0 and 150 kg N ha^{-1} and F at 150 kg N ha^{-1} (Table 3).

Table 3. Silage quality properties for each sward treatment, across both the Henfaes and North Wyke sites.

	Grass sward composition							
	R		**RC**		**F**		**FC**	
Nitrogen fertilizer addition rate (kg N ha^{-1})	0	150	0	50	0	150	0	50
DM (g kg^{-1} fresh weight)	288 ± 25	266 ± 16	268 ± 13	273 ± 16	300 ± 25	278 ± 13	289 ± 22	272 ± 20
Ash (% of DM)	6.23 ± 0.83	5.99 ± 0.82	8.05 ± 0.82	7.09 ± 0.82	7.19 ± 0.82	5.89 ± 0.83	8.27 ± 0.82	7.66 ± 0.82
Neutral detergent fibre (% of DOM)	50.0 ± 1.8	49.5 ± 1.7	43.3 ± 1.7	47.4 ± 1.7	47.7 ± 1.7	50.1 ± 1.8	44.2 ± 1.7	45.4 ± 1.7
pH (1:9 silage:water)	5.1 ± 0.1	5.0 ± 0.1	5.2 ± 0.1	5.2 ± 0.1	5.1 ± 0.1	4.9 ± 0.1	5.4 ± 0.1	5.3 ± 0.1
Total nitrogen (% of DM)	1.70 ± 0.09	1.78 ± 0.09	2.30 ± 0.09	2.20 ± 0.09	1.81 ± 0.09	1.91 ± 0.09	2.20 ± 0.09	2.30 ± 0.09
Water soluble carbohydrates (% of DM)	9.11 ± 1.99	8.75 ± 1.96	5.39 ± 1.96	4.28 ± 1.96	8.22 ± 1.96	7.88 ± 1.99	5.32 ± 1.96	4.37 ± 1.96

DM is dry matter; DOM is dry organic matter; F is *Festulolium*, FC is *Festulolium* with clover, R is ryegrass, and RC is ryegrass with clover; all values are means (n = 9) ± standard error, with the predicted means from the REML analyses presented for ash, neutral detergent fibre, pH, total nitrogen and water soluble carbohydrates. Herbage cutting dates were 30th May 2018 at the Henfaes site and 23rd May 2018 at the North Wyke site.

3.5. Biological nitrogen fixation and $\delta^{15}N$ signature of shoots

Clover $\delta^{15}N$ was consistently lower than that of the grass $\delta^{15}N$ (Figure 3), with interquartile ranges of -0.51 to +0.15 and +2.71 to +4.85‰, respectively. The proximity of clover $\delta^{15}N$ to that of the atmosphere from the zero N treatments demonstrates a strong reliance on BNF as the plant N source, with the N derived from atmosphere ranging 80 ± 1.6 to 94 ± 1.8% (± SE; n = 10) for cuts 2 to 7. However, N derived from the atmosphere was much lower in the clover from the first cut at 45 ±

13% (± SE; *n* = 9), suggesting that during clover plant establishment N was sourced from the soil as well as from BNF.

To test the hypothesis that BNF would provide a N source to the neighbouring grasses, we compared the $\delta^{15}N$ of the F and R grasses, grown under 0 N fertiliser at NW, with and without clover at every cut over the two growing seasons (Figure 3, upper and middle panels). Natural abundance ^{15}N of grass and clover shoots was examined on the 0 N treatments in the statistical analyses to avoid the impact of the N fertiliser on the $\delta^{15}N$ signature. No significant effect of clover inclusion within the sward was observed for the grass-shoot $\delta^{15}N$ values (p = 0.357), at 3.78, 4.09, 4.12 and 4.17‰ for the F, FC, R and RC treatments respectively. However, cut and the prevailing weather and seasonality associated with it was found to have a significant impact on $\delta^{15}N$ values across all swards (p < 0.001), with the lowest values of 2.43 and 2.73‰ observed at cuts performed following dry conditions, and the greatest values of 5.32 and 4.99‰ observed in the spring and autumn.

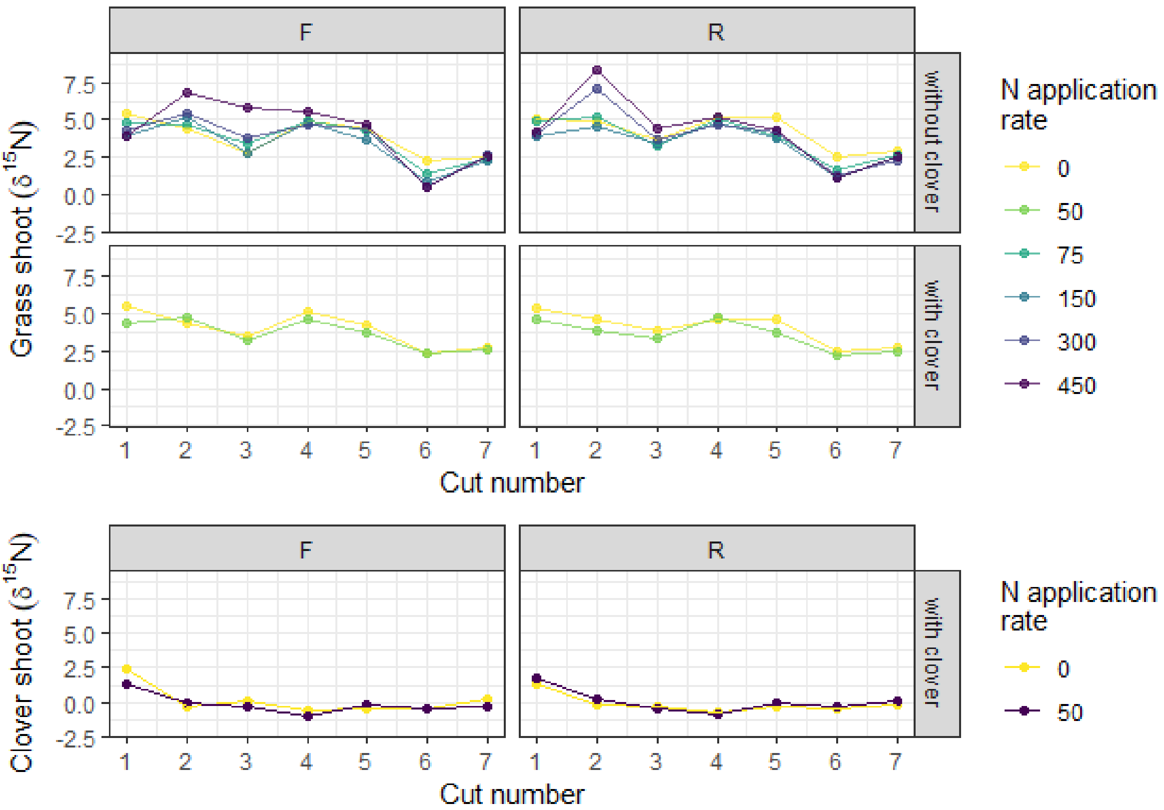

Figure 3. Shoot $\delta^{15}N$ for grass and clover at each herbage cut for the North Wyke site. Where F is the *festulolium*, R is the perennial x Italian ryegrass hybrid, cuts 1, 2, 3 and 4 were conducted in April,

June, August and October respectively in 2017, and cuts 5, 6 and 7 were conducted in May, July and September respectively in 2018**.** Data points represent mean values (*n* = 5).

3.6. Rooting and nutrient cycling at depth

Table 4. Root mass, nitrogen pools and organic carbon for the *festulolium* and ryegrass hybrid under two nitrogen fertiliser rates, at depth.

		N rate (kg N ha^{-1})	Depth (cm) 0 – 10	10 – 20	20 - 30	30 – 40	40 – 50	50 – 75	75 – 100
Root mass (g DM m^{-3} soil)	F	0	1074.7 (271.7 - 4248.6)	233.5 (55.51 - 979.4)	79 (19.8 - 313)	11.9 (2.84 - 48)	24.9 (5.76 - 105.3)	4.4 (0.86 - 19.3)	0.1 (-0.17 - 1)
		300	1546.9 (391.17 - 6115.3)	257.6 (64.97 - 1018.8)	45.7 (11.37 - 181.2)	3.1 (0.61 - 13.1)	7.0 (1.48 - 30.2)	0.8 (-0.03 - 5.1)	0.2 (-0.14 - 1.6)
	R	0	427.3 (107.93 - 1689.8)	222.4 (56.09 - 880)	32 (7.91 - 127.2)	7.9 (1.68 - 33.7)	0.7 (-0.02 - 3.8)	0.4 (-0.14 - 3.1)	0.2 (-0.15 - 1.7)
		300	603.5 (143.83 - 2529.3)	282.3 (71.23 - 1116.5)	27.3 (6.73 - 108.7)	7.2 (1.64 - 29.4)	1.4 (0.15 - 6.7)	0.7 (-0.02 - 3.3)	0.1 (-0.17 - 1.1)
Soil mineral N (mg N kg^{-1} soil)	F	0	8.42 (4.40 - 13.74)	8.44 (4.41 - 13.76)	4.27 (1.59 - 8.24)	2.14 (0.43 - 5.15)	1.42 (0.15 - 3.99)	1.18 (0.08 - 3.57)	1.05 (0.02 - 3.61)
		300	12.21 (7.23 - 18.49)	13.67 (8.37 - 20.27)	6.19 (2.83 - 10.85)	3.36 (1.06 - 6.96)	2.61 (0.64 - 5.92)	1.55 (0.17 - 4.32)	0.07 (0.68 - 1.81)
	R	0	7.93 (3.40 - 13.21)	8.20 (4.23 - 13.45)	4.35 (1.64 - 8.36)	2.59 (0.63 - 5.89)	2.50 (0.58 - 5.75)	2.86 (0.71 - 6.48)	1.13 (0 - 4.60)
		300	11.85 (6.95 - 18.04)	10.25 (5.74 - 16.05)	5.84 (2.60 - 10.38)	3.79 (1.31 - 7.58)	3.14 (0.91 - 6.70)	2.71 (0.69 - 6.06)	1.22 (0.07 - 3.75)
Soil SON (mg N kg^{-1} soil)	F	0	8.53 (2.66 - 17.74)	14.82 (7.23 - 25.11)	8.85 (3.29 - 17.1)	5.07 (1.19 - 11.64)	3.62 (0.55 - 9.39)	2.50 (0.18 - 7.51)	1.35 (0.01 - 5.79)
		300	10.60 (3.86 - 20.67)	12.10 (5.37 - 21.52)	7.56 (2.52 - 15.30)	4.13 (0.76 - 10.20)	1.52 (0.004 - 5.80)	3.02 (0.30 - 8.58)	0.08 (1.47 - 3.10)
	R	0	8.06 (2.18 - 17.63)	16.77 (8.61 - 27.63)	7.95 (2.75 - 15.84)	5.48 (1.36 - 12.35)	4.04 (0.70 - 10.14)	3.25 (0.35 - 9.09)	1.34 (0.11 - 6.98)
		300	7.26 (1.97 - 15.89)	13.18 (6.03 - 23.08)	9.06 (3.42 - 17.40)	5.85 (1.55 - 12.91)	4.15 (0.75 - 10.32)	2.59 (0.19 - 7.76)	1.35 (0.001 - 5.53)
Soil SOC (mg C kg^{-1} soil)	F	0	136.2 (51.7 - 260.9)	147.2 (62.5 - 267.6)	88.6 (26. 9 - 186.1)	70.2 (17.2 - 158.8)	55.1 (10.2 - 135.7)	36.6 (3.3 - 105.6)	45.5 (5.6 - 124.1)
		300	101.4	146.1	89.2	57.9	36.6	29.1	30.0

		(31.2 - 211.7)	(61.8 - 266.1)	(27.2 – 187.0)	(11.5 - 140.2)	(3.2 - 106.2)	(1.2 - 93.8)	(0.3 - 107.8)
R	0	90.4	148.8	92.7	74.3	55.2	30.6	24.4
		(23.8 - 199.9)	(63.6 - 269.8)	(29.2 – 192.0)	(19.0 - 165.7)	(10.1 - 136.5)	(1.4 - 97.4)	(0 - 96.9)
	300	95.7	140.5	125.5	68.0	58.0	40.5	25.1
		(28.1 - 203.5)	(57.8 - 259.4)	(48.7 - 238.1)	(16.0 - 156.3)	(11.3 - 140.9)	(4. 5 - 112.7)	(0.5 - 86.5)

F is *Festulolium*, R is ryegrass, soil mineral N is the sum of NO_3-N, NO_2-N and NH_4-N, SON is soluble organic N and SOC is soluble organic. All values are predicted means from REML analysis (95% confidence interval) across both experimental sites.

Across both sites, root mass declined with soil depth ($p < 0.001$), with a root mass maxima of 809 (confidence intervals (CI) 309.82 – 2112.9) g DM m^{-3} soil at 0 – 10 cm and minima of 0.1 (CI -0.11 – 0.8) g DM m^{-3} soil at 75 – 100 cm depth. Across all depths, root mass was greater for F than R ($p = 0.032$). In addition, there was a general trend for greater root mass at depth for F relative to R ($p = 0.038$) and the reverse was true at depths of 10 – 20 and 30 – 40 cm (Table 4). No significant effect of N supply on root mass development was observed ($p = 0.26$).

Similar trends to root mass were also observed for concentrations of soluble N forms and SOC in the soil at the rooting depths examined (Table 4). There was a significant effect of soil depth for all nutrients examined ($p < 0.001$), with greater concentrations of soil mineral N, SON and SOC at the shallower depths, and concentrations decreasing to a minimum at the 75 – 100 cm depth (Table 4). However, no significant effect of grass variety, or N fertiliser rate was observed.

4. Discussion

4.1. Impacts of grass type and red clover inclusion on herbage yields

The inclusion of red clover within the swards provided substantial N supply from BNF, with 80 – 94% of N in clover derived from the atmosphere (data from zero N fertiliser swards, Figure 3). A trend for greater DM yields and crop N offtake harvested from the mixed swards than from the single species grass swards under chemical N fertiliser at the same rates (Figures 2 and 3), was in agreement with findings from other studies for white clover (Reid et al., 1970; Burchill et al., 2014). Where chemical N fertiliser was applied to the swards containing red clover, this did not increase yields overall, although there was a general trend for increased yields for the first cut when F was the accompanying grass. Inclusion of white clover within a perennial ryegrass-sward has been shown to give a significant increase in DM yield under 0 N application, however, this effect continued for N application rates up to 200 kg N ha^{-1} (Enriquez-Hidalgo et al., 2016). Søegaard and Nielsen (2012) examined the impact of inclusion of both white and red clover within a grass sward (of *festulolium* and perennial ryegrass) and showed inclusion of red clover, at 25, 50, 75 and 100% of the clover seed, consistently increased DM yields and protein content at N application rates of 0, 110, and 220 kg N ha^{-1}. The positive effect of including red clover within the swards on yield and N offtake (Figures 2 and 3) leads us to accept the first hypothesis, that including red clover in the sward would negate the need for N fertiliser, with BNF providing N to the clover and the neighbouring grasses. However, it should be noted that the $\delta^{15}N$ dataset did not provide evidence of transfer of N from clover to neighbouring grasses. Although there is a body of evidence demonstrating the potential for improved DM yields, reduced requirements for N fertiliser, and increased protein content (Figures 2, 3, Tables 2, S3) when red clover is included within swards, there are difficulties associated with establishment and longevity of red clover. This is especially the case for red clover under grazed systems, with persistence problems and associated declines in DM yields in the third year following sowing (Eriksen et al., 2012). Persistence of twelve red clover-varieties was examined under an annual three-cut system in the UK with a general trend of improved stability of DM yield over four years observed for two of the varieties (Marshall et al., 2017), including AberClaret as used in this study. Although it was beyond the scope of this study to examine the long-term persistence of red clover, clover cover increased between the first fertiliser application and the final cut of year two, from 3.6 ± 0.6 to 36.3 ± 5.1 % of the total ground cover within the mixed swards, with greater clover coverage observed on the 0 N treatments (data from North Wyke site only; see Figure S1). However, under a rotational-grazing system in New Zealand, AberClaret was amongst the lowest performing red clover varieties in terms of percentage plant survival. These studies suggest that persistence of red clover in medium-term leys (of 3 – 4 years) and their associated benefits might be achieved. However, clarity is needed on the impacts of agronomic management (grazing *vs* cutting, or a combination of both), soil and environmental variables, and accompanying species on the persistence of red clover varieties within medium-term leys. Consequently, there is great potential for reduced N fertiliser requirements and enhanced yields of forage when red clover is included within the swards, but this is currently restricted to short-term leys and cut-grass systems.

4.2. Impacts of grass type and red clover inclusion on herbage and silage quality

A key aspect of the high-sugar grass examined here (R) was that it should provide increased carbohydrates to the rumen for energy supply (Miller et al., 2001). In this study, we observed greater ME content and lower NDF content for R relative to F in cut grass. Only when the interaction with cut was included with grass type did the grasses become significantly different from each other for all quality measures examined, which can be attributed to the impact of prevailing meteorological conditions (e.g. drought) and season on plant productivity at the time the cut was performed. However, plant establishment, especially the development of a plant root system can also be important temporally for herbage quality (McGrath, 1988). Thus, the importance of exploring the quality of herbage throughout the growing season is critical, particularly when attempting to determine whether the target phenotype is expressed under conditions applicable to agricultural systems. In line with other studies (Reid, 1970; Delevatti et al., 2019), we observed a significant trend of increasing CP content with increasing N rate on the single species swards. The same trend was observed for NDF content up to the rate of 300 kg N ha^{-1}, whilst sugar and ME content decreased with increasing N rates, as expected (McGrath 1992). The effect of increasing N rate on CP is unsurprising as N is increasingly available to the plant, however increasing N content in forage and feed is associated with greater urinary N losses from livestock (Cole et al., 2005; Dijkistra et al., 2013), and is not linked to increased livestock-N retention (Vasconcelos et al., 2009) when it is consumed in amounts surplus to requirement. Moreover, with approximately 75% and 64 - 85% of N intake excreted from beef and dairy cattle respectively (de Klein et al., 2017; Angelidis et al., 2019), increasing CP or N content of forage and feed will lead to increased N excretion with implications for NH_3 and N_2O emissions, and N leaching (Külling et al, 2001; Dijkstra et al., 2013). Additionally, increasing NDF content is linked to reduced DM intake (Vazquez and Smith, 2000), which slows growth and reduces livestock productivity, but should remain above 30% of DM (Lee et al., 2018). Therefore, N application must be at a rate that ensures DM yield and protein content and yet minimises trade-offs with sugar and ME content, as the latter two are critical for optimising animal performance (Lee et al., 2018) and minimising environmental impacts of ruminant production. In the mixed swards at 0 and 50 kg N ha^{-1}, N rate did not have a significant effect on ME, CP and NDF at p = 0.06, p = 0.09, and p = 0.23 respectively, and only significantly impacted (p < 0.001) sugar content, with greater sugar content from the 50 kg N ha^{-1} swards. This dampened response to fertiliser N is linked to BNF providing N to both the 0 and 50 kg N ha^{-1} swards and reducing the impact of N fertiliser application. The inclusion of clover within the swards was linked to greater CP and ME contents, and reduced sugar and NDF content. Therefore, the hypothesis: (2) inclusion of red clover would not detrimentally affect the herbage or silage quality can be accepted, although the proportion of clover to grass content might require optimising to ensure the herbage or silage does not contain excess N.

Some of the differences measured in the herbage quality parameters were carried through to the silage quality parameters, where clover presence within the sward resulted in greater TN and ash and reduced NDF and WSC, relative to the single species silage (Table 3). Other studies have shown that pure red clover silage has greater protein and mineral (measured through ash) content than pure grass silage (Dewhurst et al., 2003; Dewhurst, 2013). Elgersma and Søegaard (2016) also observed greater yield and ash, and lower NDF content in perennial ryegrass and red clover mixed swards relative to perennial ryegrass alone, as measured here for the clover mixed swards under 0 N fertiliser. This can be linked to the greater ash content of red clover being concentrated and the lower NDF content of red clover being diluted within a mixed sward where clover is high yielding (Elgersma and Søegaard, 2016). A key parameter for silage quality is pH, with a good fermentation resulting in pH of < 4.2 (Merry 1995). Here silage made from the single species sward and those with N fertilisation had the lowest pH values (Table 3) indicating better fermentation had occurred compared with those with clover or 0 N fertilisation. It is generally accepted that where the DM content across treatments is similar, addition of clover to a sward will result in a higher pH, because the driver for this is a combination of TN and mineral content, which buffer the acid produced during fermentation (McDonald, 1991). Additionally, increased availability of sugar is utilised by lactic acid bacteria for acid production which will reduce the pH in the silage, this process is reflected in the results seen here, with greater NDF and WSC observed in the single species swards relative to those containing clover, in the fresh (Table S2 in the supplementary information) and ensiled forage (Table 3).

Here we have shown that inclusion of red clover within short-medium term leys can be beneficial for reducing N fertiliser requirements and enhancing DM yields without detrimental effect on herbage or silage quality. However, there are concerns around inclusion of red clover within livestock diets due to its high phyto-oestrogen content, which is concentrated when it is ensiled (Marley et al., 2011). A review on the effects of legumes on ewe and cow fertility suggests that fertility issues mainly arise in breeding ewes and although these can be avoided by ensuring red clover makes up < 25% of the feed, this is difficult to achieve as foraging animals will select clover over grass (Marley et al., 2011; see also Kelly et al., 1980). The same review found contradictory evidence on the impact of red clover silage on fertility of cows and no impact on fertility was found for rams and bulls (Marley et al., 2011). Based on these findings, current recommendations in the UK are that ewes should not have red clover within their diet for the six weeks before and after copulation (AHDB, 2016).

4.3. Grass varieties for improved grassland resilience

In terms of AE_N, no differences were observed between the two grass types in their efficiency of using fertiliser N, indeed only N application rate had a significant effect on AE_N with the 150 kg N ha^{-1} rate providing optimal AEN (Table 2). The AE_N at both 75 and 150 kg N ha^{-1} was slightly greater than that reported by Egan et al. (2019) at 100 kg N ha^{-1}. However, when both the soil and fertiliser N supply was accounted for in the PFP_N a significant difference between grass types was observed, with greater efficiency of N uptake for F relative to R, thus indicating that F had an advantage over R in accessing soil N sources (Dobermann, 2005). As for AE_N, N fertiliser rate had a significant effect on PFP_N. However, PFP_N consistently decreased with increasing rate of N application (Table 2), suggesting that at low N application rates the grasses were reliant on soil N supply; over the long-term this would lead to mining of soil N (Dobermann 2005). Thus, we can reject our hypothesis (5) that F would more efficiently take up applied N than R, although it should be noted that F was better able to access endogenous soil N supply than R, which might have implications in low N input systems.

The *festulolium* variety included within this study (F) did not outperform the hybrid ryegrass (R) in terms of yield during the drought conditions experienced (Figures 1, and 2), in contrast to our third hypothesis. Tolerance to both drought and cold are key traits of the *festuca* species, which are targeted in the breeding program for *festuloliums* (Ghesquière et al., 2010). Here, F had a significantly greater root mass than R and a general trend toward greater rooting mass at depth than R (Table 4). However, the greater root mass did not equate to improved yields under the drought conditions experienced at the two field sites. As with other studies (Hejduk and Hrabě, 2003; Cougnon et al., 2017) we did not find that N significantly repressed root growth, but there was a slight trend for greater root mass from F under the 0 N rate. Thus, leading us to reject the hypothesis that enhanced rooting and nutrient cycling at depth would be observed with the *festulolium* relative to the ryegrass hybrid, but that sufficient N supply would suppress deep-rooting. Consequently, the trade-off between enhanced drought tolerance *vs.* reduced forage quality within the *festulolium* sward was not observed in this study and further research is needed to determine the role of F in yield resilience in mixed swards.

When soil N and C through the soil profile was examined to 1 m depth, we were unable to detect differences in the soil nutrient content between the F and R treatments. Indeed, a significant effect of depth was observed with greater soluble N and C concentrations in the upper soil layers, but this was true for both grass types, and agrees with other pasture-based studies (Ojeda et al., 2018). Thus, we did not find evidence to suggest enhanced N or C sequestration from the F variety examined, contradictory to suggestions that *festulolium* may have a role for increasing C capture and storage in grasslands (Humphreys et al., 2014), at least in the environments examined here. Our study was limited to one soil sampling point in the Autumn of the second growing season, although we believe root biomass would have been stable at this point, in line with the findings of Ojeda et al. (2018), it is possible that our results would have differed between sampling points.

5. Conclusion

Deep-rooting grasses have been suggested as an option for increasing C sequestration in grasslands and for increasing grassland resilience to drought events, however this was not apparent in our study and the field conditions examined here. Under the UK drought experienced in 2018, we did not observe a yield gain under the *festulolium* relative to the ryegrass hybrid. Greater root mass was observed for the *festulolium* relative to the ryegrass hybrid, however this was not found to be significant at depth (up to 1 m). The lack of deep-rooting biomass from the *festulolium* corresponded with no significant differences in soluble N and C pools at depth between the two grass varieties. Our observations support the existing evidence that red clover inclusion within short-term leys can negate the need for N fertiliser inputs, with enhanced DM yield and N offtake in the mixed swards relative to the single species grass swards. The ^{15}N natural abundance technique demonstrated that up to 94% of clover N was sourced from BNF, however we were not able to detect transfer of BNF sourced-N from clover to the accompanying grass. Inclusion of red clover within the swards resulted in greater CP and ME content and reduced sugar and NDF content of fresh herbage relative to the single species grass swards. These herbage quality parameters were typically carried through to the silage quality parameters, with greater TN and reduced NDF and WSC measured in the clover mixed swards relative to the single species grass swards. We tentatively suggest that these herbage quality differences may have implications for increased N excretion from livestock, due to greater CP and lower sugar content, and potentially for increased DM intake, through reduced NDF and greater ME content, thus sward clover content should be optimised to account for this.

The findings here demonstrate that red clover is a viable fertiliser-N replacement strategy in short-term leys and that although novel grass varieties may offer potential ecosystem services these are not always realised under field conditions. Therefore, farmers should select grass varieties based on optimising herbage quality, or perhaps look to optimise their short-term leys with a diverse range of species for enhanced grassland resilience.

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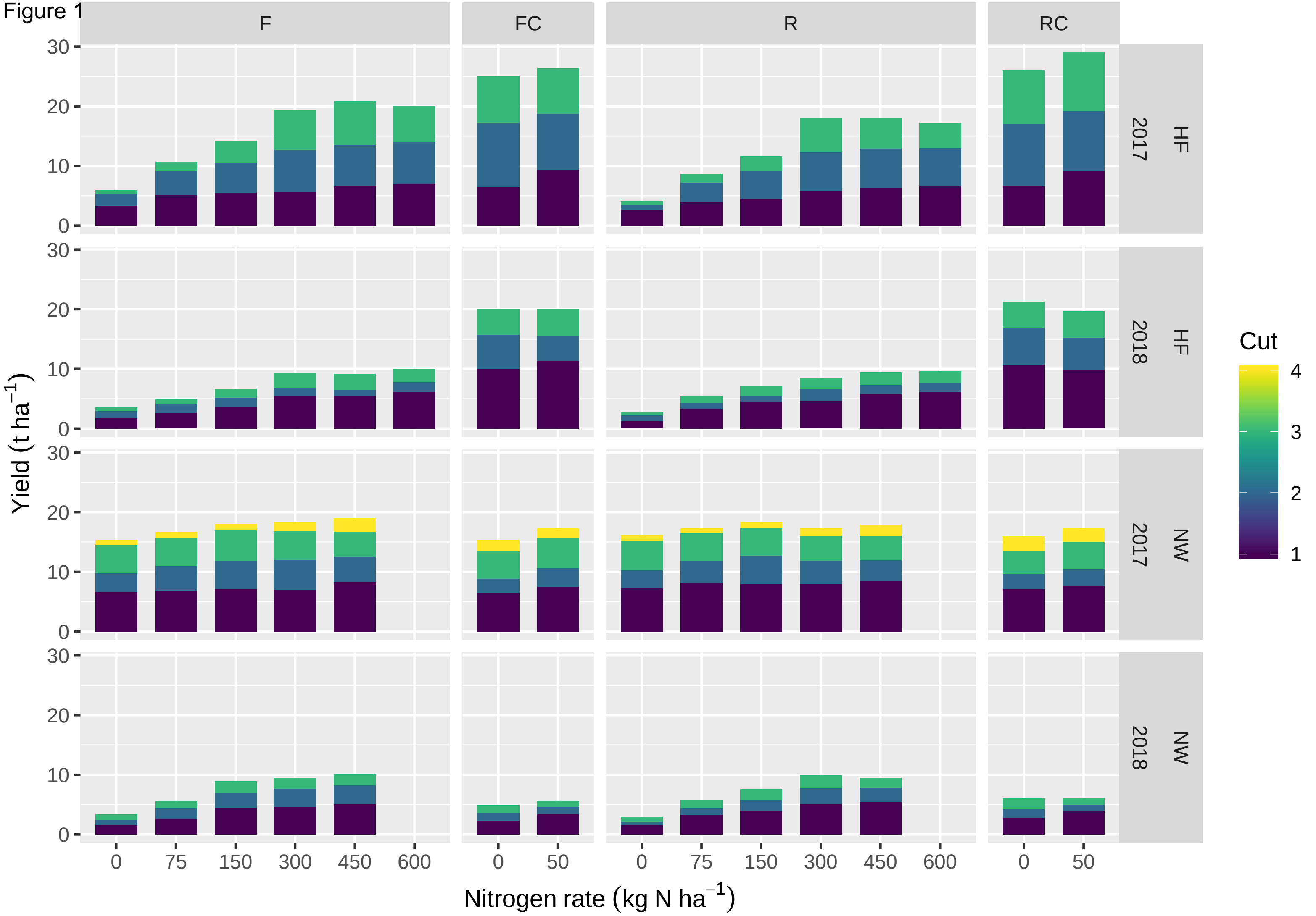
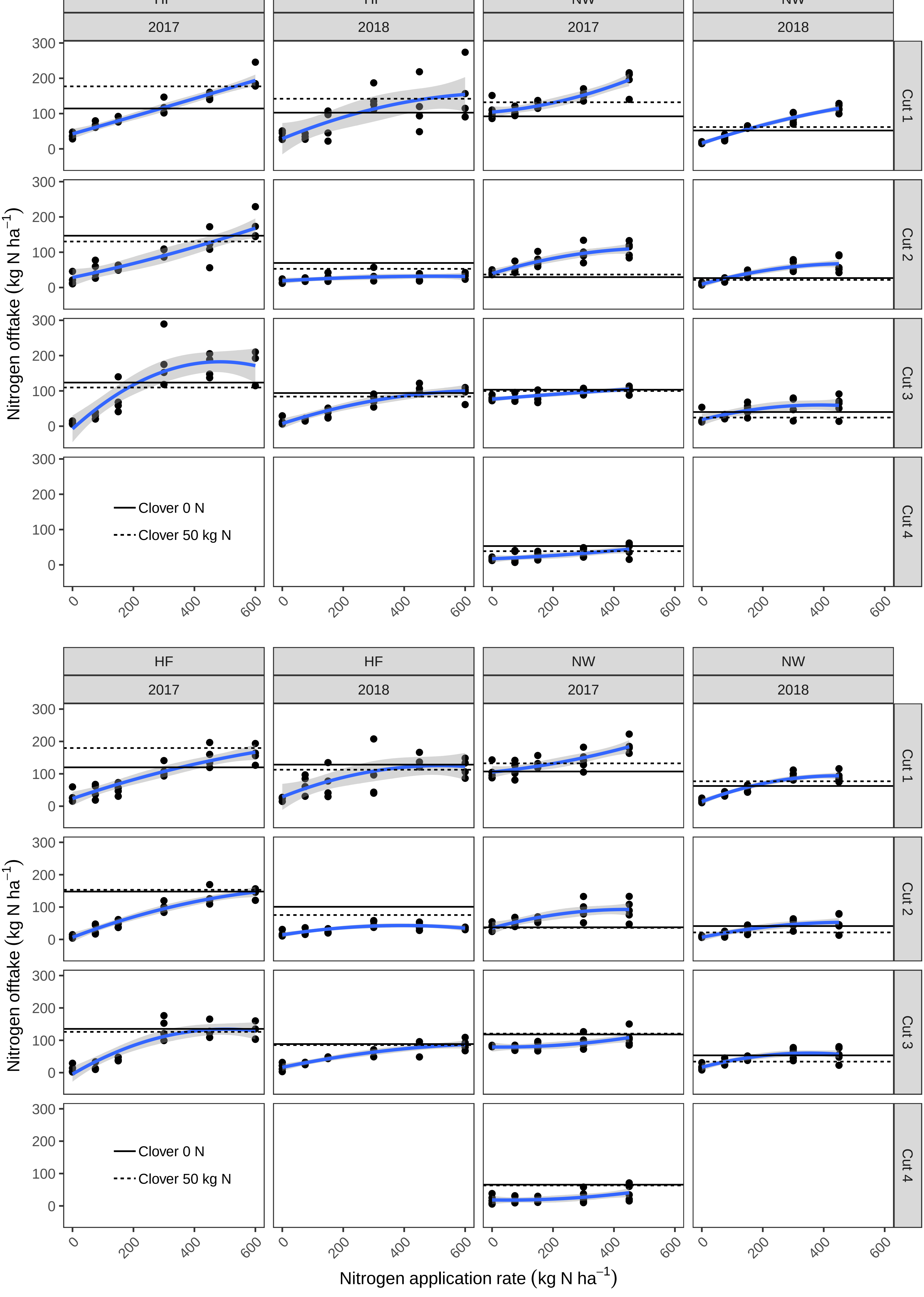
Figure 1



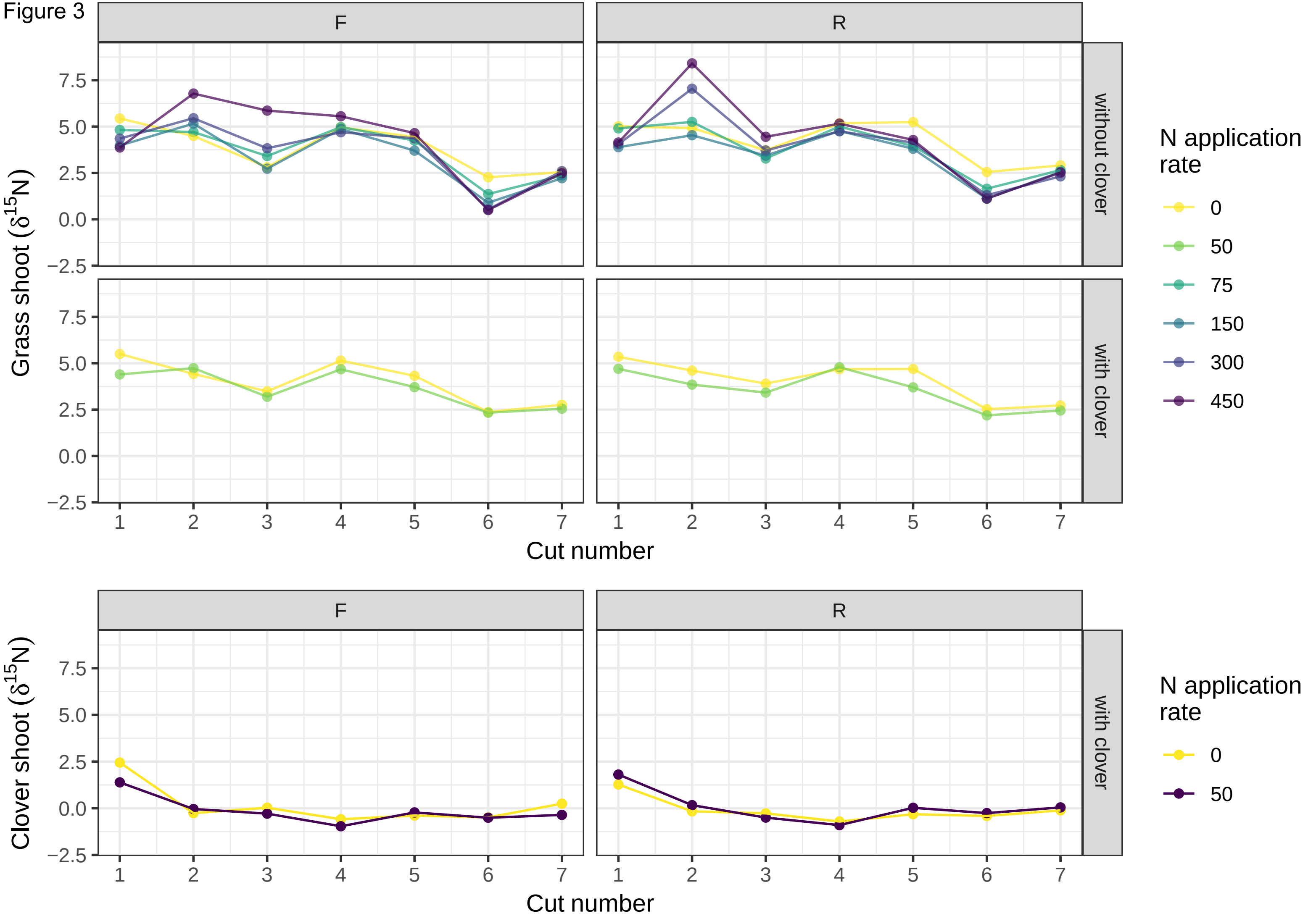
F
FC
R
RC
2017 HF
2018 HF
2017 NW
2018 NW
Cut
4
3
2
1
30
20
10
0
Yield (t ha^{-1})
0 75 150 300 450 600
0 50
Nitrogen rate (kg N ha^{-1})

Figure 2



HF
2017
HF
2018
NW
2017
NW
2018
Cut 1
Cut 2
Cut 3
Cut 4
Clover 0 N
Clover 50 kg N
Nitrogen offtake (kg N ha^{-1})
Nitrogen application rate (kg N ha^{-1})

Figure 3


F
R
without clover
with clover
Grass shoot (δ15N)
Clover shoot (δ15N)
Cut number
N application rate
0
50
75
150
300
450
7.5
5.0
2.5
0.0
−2.5
1
2
3
4
5
6
7

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure S1