Endophyte mixture effects on ryegrass staggers in sheep

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Abstract

Epichloë endophytes protect ryegrass from insect pests, but no strain provides both the highest level of insect protection and lowest risk to livestock health. This study examined whether mixtures of endophytes could improve animal safety by mitigating ryegrass staggers. At Courtenay, Canterbury, pastures varying widely in sown proportions of perennial ryegrass infected with nea3, nea12 and Standard Endophyte (SE) were sown in May 2019 and grazed by lambs in summer 2020 and hoggets in spring 2020. In summer, pastures sown with at least 65% nea3, up to 35% nea12 and no SE delayed severe staggers (score 4–5) by 1 week. These pastures had low concentrations of tremorgenic alkaloids: epoxyjanthitrem I (≤0.28 ppm) synthesised by nea12 and paxilline (≤0.15 ppm) and terpendole C (≤1.37 ppm) of nea3. In spring, pastures sown with at least 65% nea3 and no more than 35% nea12 and/or SE did not induce staggers (score 0–0.1). Those pastures had low concentrations of the potent tremorgen lolitrem B (≤0.30 ppm) of SE, epoxyjanthitrem I (≤0.39 ppm) of nea12 and paxilline (≤0.09 ppm) and terpendole C (≤0.41 ppm) of nea3 and SE. Therefore, pastures sown with ≥65% nea3 and <35% nea12 minimised staggers in both seasons.

Keywords: alkaloid, diversity, Epichloë, Lolium perenne, simplex design

Introduction

Perennial ryegrass (*Lolium perenne* L.) is a standard ingredient of pastures in New Zealand (Stewart et al. 2022). In most situations the ryegrass is infected with an asexual *Epichloë* fungal endophyte. This endophyte has several strains that produce a range of alkaloids, including lolitrem B, peramine, ergovaline and epoxyjanthitrems, inside the host plant. The alkaloids protect the ryegrass from a range of important insect pests (Popay and Hume 2011), but some can also cause ryegrass staggers, productivity losses and heat stress in livestock (Fletcher 1999). The positive effects on pests and negative effects on livestock vary among strains with no single strain providing both the highest level of insect protection and the lowest risk to livestock health (Caradus et al. 2021). A pasture made up of two or more strains with a diverse alkaloid profile that provided both outcomes would be a valuable proposition on farms where insect pests challenge ryegrass persistence.

There is limited published evidence about the impacts of endophyte mixtures on livestock health. In Logan et al. (2015), perennial ryegrass with a mixture of nea2 and nea6 strains (marketed as NEA2; Eady 2021), a mixture of nea2 and nea3 strains (marketed as NEA4) and nea3 by itself, all resulted in similar or better growth rates and less ryegrass staggers in lambs and hoggets than with Standard Endophyte (SE) (nea2, nea3, nea6 and SE are all *E. festucae* var. *lolii*). Similarly, Fletcher et al. (2017) showed that perennial ryegrass with a mixture of nea2 and nea6 (NEA2) caused less severe staggers in lambs than SE. These results can be explained by the effects of strain identity and diversity. The diversity effect is defined as the excess of mixture performance over that expected from the components' monoculture performances, called the identity effect (Black et al. 2017). The diversity effect is the aggregate of interactions that might occur among components in a pasture. The contribution of strain identity effects and any strain interactions to animal health would be scaled by the relative proportions of strains in the pasture. For example, NEA2 and NEA4 would have caused less staggers than SE because nea2, nea3 and nea6 had lower identity effects on staggers than SE and were present at lower proportions in the NEA2 and NEA4 mixtures than SE was in the SE monoculture. Only nea2 and SE produced lolitrem B that causes ryegrass staggers. Therefore, the effect of strain proportion on ryegrass staggers will be a key factor determining the formulation of optimal endophyte mixtures for improved animal safety.

The aim of this study was to formulate perennial ryegrass-endophyte pastures that mitigate ryegrass staggers for sheep. This involved three objectives: 1) quantify the influence of sown proportions of three strains – nea3, nea12 (*Epichloë* sp. LpTG-3) and SE – on staggers, 2) predict the staggers responses to any combination of the endophyte proportions and 3) identify the optimal formulations that minimise the risk of staggers.

Materials and Methods

An endophyte mixture experiment was carried out at the Barenbrug Plant Breeding Station, Courtenay in Canterbury, New Zealand (43°27'32"S, 172°10'55"E, 190 m elevation, 755 mm mean annual rainfall). The

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site was 4 ha of flat agricultural land divided into four equally-sized paddocks (A–D). Crop history was 3.5–4 years of ryegrass pasture for all paddocks and then 6 months of ryecorn (*Secale cereale* L.) cv. Amilo for paddocks C–D. Soil type was Templeton silt loam with a plant-available water capacity of 110 mm to 0.6 m depth. Soil fertility was pH 6.2, Olsen P 25 mg/kg, K 0.28 mEq/100 g, SO_4-S 7.5 mg/kg, Mg 0.44 mEq/100 g, Ca 7.5 mEq/100 g and Na 3.9 mEq/100 g to 0.15 m depth on 5 June 2020.

The three strains were chosen for their diverse alkaloid profiles that were beneficial to insect protection. The nea3 strain was known to produce peramine and ergovaline, nea12 produces epoxyjanthitrems in similar concentrations to AR37 (Spangenberg et al. 2016) and SE produces lolitrem B, peramine and ergovaline in high concentrations (Fletcher 1999; Caradus et al. 2021). The strains were tested in perennial ryegrass cv. Maxsyn.

Seven combinations of three strain proportions (p_1–p_3) were identified as test points in a simplex centroid design (Cornell 2002). The design space was left unrestricted to allow monocultures and mixtures of the strains, such that $0 \le p_i \le 1$ and $\Sigma_{i=1}^{S} p_i = 1$ where S is the number of strains and p_i is the initial proportion of strain i. There were three monocultures, three binary mixtures (1/2 of each of two strains) and a centroid mixture (1/3 of each strain). A monoculture of endophyte-free (nil) Maxsyn was also necessary for the animal testing as a negative control.

The endophyte levels were randomly allocated to plots within each of three replicate blocks. Plot layout comprised two rows of 12 plots with one row across paddocks A–B, one row across paddocks C–D and two rows of four adjacent plots in each block. Plot area was 0.156 ha and plot dimensions averaged 31 m by 50.3 m. A lane along the south side of each row connected each plot to a corral at the east side of the experiment.

The site was sprayed out and then cultivated into a seedbed between November 2018 and May 2019. Fertiliser was applied on 26 April 2019 (Cropzeal 16N, 15% N, 8% P, 10% K and 9% S at 250 kg/ha). Plots were sown on 14–16 May 2019 using a precision drill with coulters spaced 15 cm apart. Sowing rate was held constant at 685 seeds/m^2 (13.7 kg/ha).

Seed germination was 95, 91, 85 and 87% (14 days incubation at 20°C) and seed endophyte infection was 84, 92, 99 and 0% (seed squash method; Latch et al. 1987) for nea3, nea12, SE and nil endophyte respectively. Immunoblot analysis of 30 tillers/plot (Eady 2021) on 19 November 2019 showed that the ryegrass was infected with endophyte at nea3 83%, nea12 80%, SE 100%, nea3-nea12 87%, nea3-SE 97%, nea12-SE 94%, nea3-nea12-SE 91% and nil 0%. Endophyte typing (KASP; Eady 2021) of the sampled tillers showed infections at nea3 80%, nea12 73%, SE 100%, nea3-nea12 54%-30%, nea3-SE 43%-53%, nea12-SE 49%-33% and nea3-nea12-SE 37%-11%-41% respectively.

Permission, for all procedures involving sheep, was granted by the Lincoln University Animal Ethics Committee (AEC; 2020-03 and 2020-18).

An initial animal test aimed to create a worst-case scenario for endophyte toxicity in summer 2020. Plots were cut for baleage on 1 November 2019 and then closed for 94 days with neither irrigation nor fertiliser to accumulate 5–6 t dry matter (DM)/ha above ground level. Plots were then fenced and fitted with water troughs. Two hundred and forty lambs – female, Suffolk × Perendale cross, 5–6 months of age and 32.5 ± 0.1 kg live weight (fasted) – were randomly assigned to the plots (10 lambs/plot) on 3 February 2020. The test ceased on 3 March 2020. Rainfall was 128 mm from 1 November 2019 to 29 February 2020.

A repeat animal test aimed to create a low endophyte toxicity scenario in spring 2020. Plots were cleared of internal fences, grazed by sheep down to 1–1.5 t DM/ha (above ground level) on 5–6 March 2020, cut for baleage on 27 July 2020 and then re-fenced. Fertiliser was applied on 12 June 2020 (Nrich SOA, 20.5% N and 23% S at 100 kg/ha), 7 July 2020 (Serpentine Super 15K, 4.8% P, 15% K and 5.9% S at 500 kg/ha) and 19 August 2020 (SustaiN, 46% N at 120 kg/ha). Irrigation was applied on 20 August 2020 (15 mm) and 22 October 2020 (30 mm). One hundred and sixty-eight hoggets – female, Romney, 12–13 months of age, 40.0 ± 0.1 kg live weight (fasted) and randomised (seven hoggets/plot) – grazed from 14 September to 10 December 2020. Rainfall for 1 September–31 December was 190 mm.

Lambs were checked for ryegrass staggers daily and, upon the first sign, scored each week. The lambs were run for 400 m from their plot to the corral where each lamb was scored for staggers on a 0–5 ascending scale (Keogh 1973). Lambs with severe staggers (score 4–5), and any group with just one remaining lamb, were removed from the experiment, and the others were returned to their plots. The score of a removed animal carried over to the next observation date.

Herbage in a 0.53 m × 5.0 m strip was cut at 40–50 mm height using a lawn mower at two sites per plot every 2 weeks. The clippings were weighed and a subsample of about 100 g was weighed, dried at 90°C for 1–2 days and re-weighed to determine herbage mass (above 40–50 mm). A second subsample of about 100 g was freeze dried at –30°C and milled through a 1-mm sieve for alkaloid and nutritive analyses every 2–4 weeks. Herbage at 10 random sites per plot was cut at 10–20 mm height, mixed, separated into leaf, stem and dead material, dried at 65°C for 1–2 days and weighed

Table 1 Model coefficient estimates (Coef.), standard errors (SE) and significance levels (Sig.) for ryegrass staggers at 0, 1 and 2 weeks after the first symptom in summer (from 11 February) and spring (from 23 November) 2020.

		Summer			Spring		
Weeks	**Term**	**Coef.**	**SE**	**Sig.**	**Coef.**	**SE**	**Sig.**
0	β_1	0.796	0.382	***	-0.018	0.255	***
0	β_2	2.196	0.382	***	0.077	0.255	***
0	β_3	4.296	0.382	***	1.458	0.255	***
0	δ_{12}	-4.72	1.76	*	0.17	1.03	
0	δ_{13}	2.28	1.76		-2.59	1.03	*
0	δ_{23}	4.55	1.76	*	1.98	1.03	+
1	β_1	3.113	0.22	***	0.002	0.214	***
1	β_2	4.013	0.22	***	0.383	0.214	***
1	β_3	4.379	0.22	***	1.716	0.214	***
1	δ_{12}	-1.39	1.01		-0.805	0.984	
1	δ_{13}	3.61	1.01	**	-3.091	0.984	**
1	δ_{23}	2.08	1.01	+	0.338	0.984	
2	β_1	4.071	0.119	***	0.008	0.191	***
2	β_2	4.237	0.119	***	0.77	0.191	***
2	β_3	4.404	0.119	***	1.627	0.191	***
2	δ_{12}	0.253	0.545		-1.683	0.878	+
2	δ_{13}	1.253	0.545	*	-2.635	0.878	**
2	δ_{23}	1.186	0.545	*	1.175	0.878	

β_1–β_3 are the identity effects of nea3, nea12 and standard endophytes respectively. δ_{12}, δ_{13} and δ_{23} are interaction terms for pairs of strains. Significance codes: *** P<0.001; ** 0.001<P<0.01; * 0.01<P<0.05; + 0.05<P<0.1.

to determine botanical composition every 2 weeks.

The alkaloid analysis tested for concentrations of peramine, ergovaline, lolitrem B, epoxyjanthitrem I, paxilline and terpendole C in the herbage DM using high-performance liquid chromatography – mass spectrometry (HPLC-MS; Hettiarachchige et al. 2019). Nutritive value was determined as crude protein (CP) and metabolisable energy (ME) by near infrared spectroscopy (FOSS NIRSystems II 5000).

A pairwise diversity-interaction model (Kirwan et al. 2009) was fitted to ryegrass staggers score (average/plot) separately at 0, 1 and 2 weeks upon the first sign of staggers. The model was also fitted to alkaloid concentrations at the closest sampling date to the onset of staggers. The model assumes a unique interaction effect for each pair of strains. The model equation is:

$$y = \sum_{i=1}^{S=3} \beta_i p_i + \sum_{\substack{i,j=1 \\ i<j}}^{S=3} \delta_{ij}(p_i p_j) + \epsilon$$

where y is a pasture function response variable (e.g., staggers score), S is the number of strains and p_i is the sown proportion of perennial ryegrass with strain i. β_i scaled by p_i is the expected contribution of strain i to the response and is referred to as the identity effect of strain i. δ_{ii} is the coefficient of the interaction between strains i and j and is scaled by the product of p_i and p_j to determine the expected contribution of the interaction to the response. The error term, ϵ, is assumed to be normally distributed with constant variance. The model was fitted in Minitab software. The response optimisation function in Minitab was used to find the optimal strain or mixture of strains that minimised ryegrass staggers score. Pastures with endophyte and nil endophyte were compared by one-way analysis of variance with contrasts.

Results

The model coefficients revealed significant identity and interaction effects of endophyte strains on ryegrass staggers score (Table 1). In summer, nea3 had the lowest identity effect at 0 weeks (0.8) and 1 week (3.1) upon the first sign of staggers on 11 February, but all three strains had high identity effects at 2 weeks (4.1–4.4).

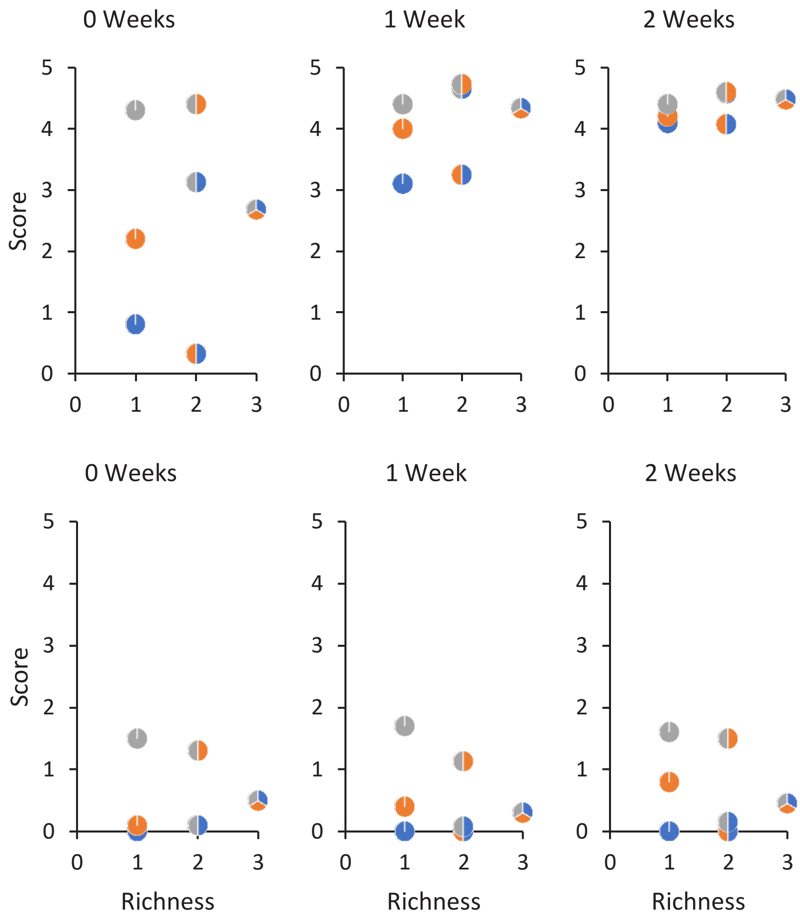

Figure 1 Predicted ryegrass staggers score (0–5 scale) in response to sown richness (number of strains) and proportions of perennial ryegrass with nea3 (blue), nea12 (orange) and standard (grey) endophytes at 0, 1 and 2 weeks after the first symptom in summer (from 11 February; top panels) and spring (from 23 November; bottom panels) 2020.

Therefore, without considering the interaction terms, maximising the proportion of nea3 in the pasture would minimise ryegrass staggers within the first week.

However, the interaction term δ_{12} was negative and significant ($P<0.05$) at 0 weeks, and one or both interaction terms δ_{13} and δ_{23} were positive and significant ($P<0.05$) at each date. These results implied that nea3 and nea12 had a synergistic relationship and pairs of either nea3 or nea12 and SE had antagonistic relationships. Therefore, the inclusion of nea12 in the pasture would also be beneficial.

In spring, nea3 had the lowest identity effect followed by nea12 and SE at 0, 1 and 2 weeks upon the first sign of staggers on 23 November (average scores across dates: $0<0.4<1.6$; Table 1). The interaction between nea3 and SE was also negative and significant ($P<0.05$) at each date, so combining these two strains was important. The nil endophyte control had no ryegrass staggers.

To predict ryegrass staggers score from the models for a monoculture of nea3 on 11 February (0 weeks) would result in: $\hat{y} = 0.8 \times 1 + 2.2 \times 0 + 4.3 \times 0 – 4.7 \times 1 \times 0 + 2.3 \times 1 \times 0 + 4.6 \times 0 \times 0 = 0.8$. To predict for an equi-proportional mixture of the three strains on 11 February: $\hat{y} = 0.8 \times 1/3 + 2.2 \times 1/3 + 4.3 \times 1/3 – 4.7 \times 1/3 \times 1/3 + 2.3 \times 1/3 \times 1/3 + 4.6 \times 1/3 \times 1/3 = 2.7$.

The predicted staggers scores are plotted against levels of sown number (richness) and proportions of perennial ryegrass with nea3, nea12 and SE in Figure 1. The predicted monoculture (richness level 1) scores are equal to the identity effects (Table 1). In summer, the 50-50 mixture of nea3 and nea12 had a lower score than nea3 at 0 weeks (0.3 vs. 0.8), due to the negative interaction effect between nea3 and nea12 (Table 1), and a similar score to nea3 at 1 (3.3 vs. 3.1) and 2 (4.1) weeks. However, the high identity effect of SE had a big impact on the scores for mixtures with SE at each date. In spring, the 50-50 mixtures of nea3 and either nea12 or SE had similar scores to nea3 across all three dates (0–0.2 vs. 0), due to their negative interaction effects (Table 1).

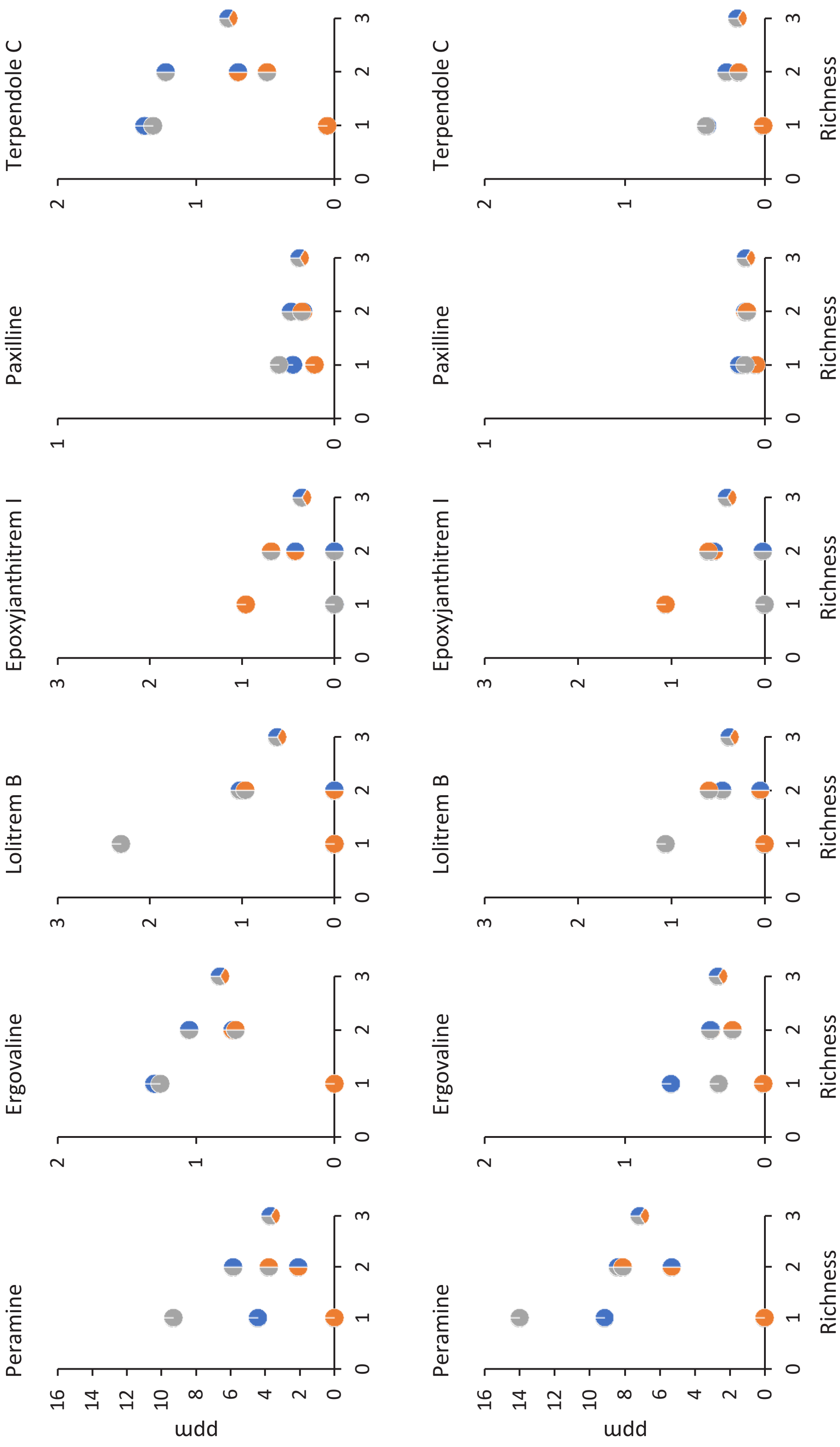

Figure 2 Predicted alkaloid concentrations in response to sown richness (number of strains) and proportions of perennial ryegrass with nea3 (blue), nea12 (orange) and standard (grey) endophytes in summer (5 February; top panels) and spring (11 November; bottom panels) 2020.

We defined the optimal endophyte mixture as one that would minimise the onset of ryegrass staggers in a high toxicity scenario in summer. The optimisation analysis of predicted scores on 11 February showed that the best formulation was a 65-35 mixture of nea3 and nea12. The predicted score for this mixture was 0.2, 3.1 and 4.1 at 0, 1 and 2 weeks respectively in summer and 0–0.1 across all three dates in spring. However, a wide range of combinations between the nea3 monoculture and the 65-35 mixture of nea3 and nea12 also gave minimum predictions (0–0.1).

The strains had significant identity effects ($P<0.001$) but no significant interaction effects on alkaloid concentrations. Therefore, only the identity effects scaled by the strains' proportions affected the alkaloid profiles of the pastures (Figure 2). For example, for peramine in summer, nea3 was lower than SE (4.43 vs. 9.29 ppm) and the 50-50 mixture of nea3 and SE was about halfway between these two strains (5.83 ppm). Looking at the other alkaloids for monocultures, nea3 and SE were similar for ergovaline (1.30 vs. 1.26 ppm), only SE had lolitrem B (2.31 ppm), only nea12 had epoxyjanthitrem I (0.96 ppm) and nea3 and SE were higher than nea12 for paxilline (0.15 and 0.20 vs. 0.07 ppm) and terpendole C (1.37 and 1.31 vs. 0.05 ppm). In spring, nea3 was lower than SE for peramine (9.12 vs. 14 ppm), nea3 was higher than SE for ergovaline (0.67 vs. 0.33), SE had 1.07 ppm of lolitrem B, nea12 had 1.06 ppm of epoxyjanthitrem I and nea3 and SE were similar for paxilline (0.09 and 0.07 ppm) and terpendole C (0.41 and 0.42 ppm).

Herbage mass and composition were generally similar across endophyte levels. In summer, average (± standard error) herbage mass was 3.7 ± 0.10 and 2.9 ± 0.11 t DM/ha on 5 and 20 February respectively, and greater ($P<0.001$) with endophyte than nil endophyte on 20 February (3.0 ± 0.10 vs. 1.9 ± 0.19 t DM/ha). Averaged across dates, DM content was 60 ± 0.9%, leaf 5 ± 0.4%, stem 68 ± 1.3%, dead 28 ± 1.1%, CP 4.6 ± 0.1% and ME 8 ± 0.1 MJ/kg DM. In spring, average herbage mass was 1.6 ± 0.06, 1.8 ± 0.07, 0.6 ± 0.04, 0.9 ± 0.05 and 0.4 ± 0.05 t DM/ha on 14 September, 29 September, 11 November, 25 November and 9 December respectively. Averaged over time, DM content was 29 ± 0.4%, leaf 43 ± 1.9%, stem 20 ± 1.3%, dead 37 ± 0.9%, CP 14 ± 0.3% and ME 11 ± 0.1 MJ/kg DM. Leaf content was greater ($P<0.01$) with endophyte than nil endophyte on 25 November (25 ± 1.3% vs. 12 ± 7.0%).

Discussion

The rapid onset of severe ryegrass staggers (score 4–5) in summer emphasised the potential risks to livestock health of all three strains when growing conditions result in high endophyte toxicity. nea3 had the lowest identity effect on staggers (Table 1), so maximising its proportion in the pasture was important (Figure 1). However, nea3 and nea12 interacted synergistically with one another, meaning the inclusion of nea12 was also beneficial. Therefore, the wide range of strain proportions between the nea3 monoculture and the 65-35 mixture of nea3 and nea12 delayed severe staggers for 1 week. From a practical perspective, a week without severe staggers after entry into the pasture would provide enough time to move livestock to a safe pasture (e.g., nil endophyte ryegrass), should on-farm conditions be as extreme as our experiment.

The alkaloid profiles of the pastures were explained by the strain identity effects scaled by their sown proportions (Figure 2). This helped to explain the dependence of ryegrass staggers on strain proportions (Figure 1). nea3 induced lower staggers than nea12 followed by SE because paxilline and terpendole C were less potent, or present in lower concentrations, than epoxyjanthitrem I of nea12, which was less potent than lolitrem B of SE. Epoxyjanthitrems are known to induce lower staggers than lolitrem B (Fletcher 2005; Finch et al. 2020). Paxilline and terpendole C are related to lolitrem B, but their tremorgenicity is different and may not manifest in staggers for sheep (Gallagher et al. 1977; Fletcher et al. 1993; Munday-Finch 1997). Ergovaline is not tremorgenic (Klotz and Nicol 2016) and peramine has no known effects on livestock (Fletcher 1999). Therefore, additional unidentified tremorgens could have also induced the staggers for nea3.

The negative interaction term for nea3 and nea12 on staggers in summer (Table 1) implied that these two strains interacted synergistically with one another. This interaction was at a magnitude that decreased staggers more than the weighted average of the two strain's identity effects (Figure 1). The two strains appeared to benefit from each other's presence, but the explanation was more likely a dose response to their tremorgenic alkaloids (Fletcher and Easton 1997; Finch et al. 2018). For example, the 65-35 mixture of nea3 and nea12 induced almost no staggers (0.2) on 11 February (0 weeks) because its predicted concentration of epoxyjanthitrem I (0.96 for nea12 × 0.35 = 0.28 ppm) was insufficient to induce tremors. The seed and tiller analyses also indicated that the pastures contained some plants with no endophyte, which would have added to the dilution effect of nea3 on staggers.

The positive interaction terms for nea3 or nea12 and SE in summer (Table 1) implied that these pairs of strains had antagonistic interactions that increased staggers above the levels predicted solely from the identity effects (Figure 1). However, the explanation points more towards the dose response to their tremorgens. The 50-50 mixture of nea12 and SE, for example, had predicted concentrations of lolitrem B (0.97 ppm) and epoxyjanthitrem I (0.68 ppm) that must have resulted in threshold concentrations for severe staggers in the grazing lambs (Galey et al. 1991; di Menna et al. 2012; Fletcher et al. 2017).

The incidence of mild staggers for the SE monoculture in spring (a peak score of 1.7; Figure 1) rendered this animal test as invalid according to Endophyte Technical Committee protocols as a minimum score of 2.3 for SE was required. The repeat test created conditions conducive to low toxicity as alkaloid concentrations are usually lower in spring compared to summer and autumn (Thom et al. 2013). Under these conditions, nea3 was consistently more important than the other two strains to achieve zero staggers (Table 1). The negative interaction terms for nea3 and SE also suggested that the presence of nea3 in the pastures had a dilution effect that mitigated staggers (Fletcher et al. 2017). Pastures with at least 65% nea3 did not induce staggers because their concentrations of lolitrem B (≤0.30 ppm), epoxyjanthitrem I (≤0.39 ppm), paxilline (≤0.09 ppm) and terpendole C (≤0.41 ppm) were too low for clinical staggers. Therefore, the combined evidence from the initial and repeat tests showed that sown proportions of perennial ryegrass with at least 65% nea3, up to 35% nea12 and no SE mitigated ryegrass staggers.

The mixture experiment showed that mixed endophyte populations can reduce potential ryegrass staggers that occur in some monocultures of endophytes. The diversity-interaction modelling approach (Kirwan et al. 2009) provided a predictive tool to evaluate endophyte mixtures beyond those included in the experimental design, thus keeping the number of entries in the animal tests to a minimum. Care should be taken when interpreting the ryegrass staggers scores since the scoring scale (Keogh 1973) is ordinal and non-linear. A score of 2, for example, while representing less severe staggers than a score of 4, does not represent half the staggers.

Further research should test for mixture effects on other livestock responses, including growth rate and core temperature, which are sensitive to ergovaline (Layton et al. 2004; Fletcher et al. 2017). A multi-variate analysis of staggers, growth rate and core temperature might alter the optimal endophyte mixtures. Further studies may include environmental or management factors. For example, the alkaloids for SE may confer greater benefits in environments with high abiotic stressors such as drought and insect herbivory (Hewitt et al. 2021). The work should also examine mixtures for changes in relative abundances of endophytes and nil endophyte in pastures over time, examine the effects of endophyte proportions on insect challenges, including our optimal mixtures for minimum staggers, and confirm whether alkaloid profiles are consistent between herbage offered to and ingested by grazing animals.

Conclusions

The sown proportions of perennial ryegrass with nea3, nea12 and SE affected ryegrass staggers in lambs and hoggets. nea3 induced the lowest staggers because paxilline and terpendole C of nea3 were either less potent, or present in lower concentrations, than epoxyjanthitrem I of nea12 and lolitrem B, paxilline and terpendole C of SE. Severe staggers in the absence of lolitrem B or epoxyjanthitrems demonstrated the need to consider other tremorgenic alkaloids, known and unknown, when assessing endophyte safety. Inclusion of nea3 in mixtures with nea12 and SE had a dilution effect on staggers. The optimal endophyte formulations that minimised staggers in our experiment were at least 65% nea3, up to 35% nea12 and no SE.

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REFERENCES

Black AD, Anderson S, Dalgety SK. 2017. Identification of pasture mixtures that maximise dry matter yield. *Journal of New Zealand Grasslands 79*: 103-109. https://doi.org/10.33584/jnzg.2017.79

Caradus JR, Chapman DF, Cookson T, Cotching B, Deighton MH, Donnelly L, Ferguson J, Finch SC, Gard S, Hume DE, Johnson LJ, Kerr GA, Norriss MG, Peddie K, Popay AJ. 2021. *Epichloë* endophytes – new perspectives on a key ingredient for resilient perennial grass pastures. *Resilient Pastures Symposium. Grassland Research and Practice Series 17*: 347-360. https://doi.org/10.33584/rps.17.2021.3435

Cornell JA. 2002. *Experiments with mixtures: designs, models, and the analysis of mixture data*. 3rd edition. Wiley, Chichester, United Kingdom. 305 p. https://doi.org/10.1002/9781118204221

di Menna ME, Finch SC, Popay AJ, Smith BL. 2012. A review of the *Neotyphodium lolii / Lolium perenne* symbiosis and its associated effects on animal and plant health, with particular emphasis on ryegrass staggers. *New Zealand Veterinary Journal 60*: 315-328. https://doi.org/10.1080/00480169.2012.697429

Eady C. 2021. The impact of alkaloid-producing *Epichloë* endophyte on forage ryegrass breeding: a New Zealand perspective. *Toxins 13*: 158. https://doi.org/10.3390/toxins13020158

Finch SC, Prinsep MR, Popay AJ, Wilkins AL, Webb NG, Bhattarai S, Jensen JG, Hawkes AD, Babu

JV, Tapper BA, Lane GA. 2020. Identification and structure elucidation of epoxyjanthitrems from *Lolium perenne* infected with the endophytic fungus *Epichloë festucae* var. *lolii* and determination of the tremorgenic and anti-insect activity of epoxyjanthitrem I. *Toxins 12*: 526. https://doi.org/10.3390/toxins12080526

Finch SC, Vlaming JB, Sutherland BL, van Koten C, Mace WJ, Fletcher LR. 2018. Ergovaline does not alter the severity of ryegrass staggers induced by lolitrem B. *New Zealand Veterinary Journal 66*: 93-97. https://doi.org/10.1080/00480169.2017.1416693

Fletcher LR, Finch SC, Sutherland BL, deNicolo G, Mace WJ, van Koten C, Hume DE. 2017. The occurrence of ryegrass staggers and heat stress in sheep grazing ryegrass-endophyte associations with diverse alkaloid profiles. *New Zealand Veterinary Journal 65*: 232-241. https://doi.org/10.1080/00480169.2017.1329673

Fletcher LR. 1999. "Non-toxic" endophytes in ryegrass and their effect on livestock health and production. *Ryegrass endophyte: an essential New Zealand symbiosis. Grassland Research and Practice Series 7*: 133-139. https://doi.org/10.33584/rps.7.1999.3393

Fletcher LR. 2005. Managing ryegrass-endophyte toxicoses. In: Roberts CA, West CP & Spiers DE. Eds. *Neotyphodium in cool-season grasses*. Hoboken, NJ: Blackwell Publishing, pp. 229-241. https://doi.org/10.1002/9780470384916.ch10

Fletcher LR, Easton HS. 1997. The evaluation and use of endophytes for pasture improvement. In: Bacon CW & Hill NS. Eds. *Neotyphodium/Grass Interactions*. Boston, MA: Springer, pp. 209-227. https://doi.org/10.1007/978-1-4899-0271-9_39

Fletcher LR, Garthwaite I, Towers NR. 1993. Ryegrass staggers in the absence of lolitrem B. *Proceedings of the Second International Symposium on Acremonium/Grass Interactions*: 119-121.

Galey FD, Tracy ML, Craigmill AL, Barr BC, Markegard G, Peterson R, O'Connor M. 1991. Staggers induced by consumption of perennial ryegrass in cattle and sheep from northern California. *Journal of the American Veterinary Medical Association 199(4)*: 466-470.

Gallagher RT, Keogh RG, Latch GCM, Reid CSW. 1977. The role of fungal tremorgens in ryegrass staggers. *New Zealand Journal of Agricultural Research 20(4)*: 431-440. https://doi.org/10.1080/00288233.1977.10427356

Hettiarachchige IK, Elkins AC, Reddy P, Mann RC, Guthridge KM, Sawbridge TI, Forster JW, Spangenberg GC. 2019. Genetic modification of asexual *Epichloë* endophytes with the perA gene for peramine biosynthesis. *Molecular Genetics and Genomics 294(2)*: 315-328. https://doi.org/10.1007/s00438-018-1510-x

Hewitt KG, Matthew C, McKenzie CM, Mace WJ, Popay AJ. 2021. The role of *Epichloë* grass endophytes during pasture renewal. *Resilient Pastures Symposium. Grassland Research and Practice Series 17*: 339-346. https://doi.org/10.33584/rps.17.2021.3446

Keogh RG. 1973. Induction and prevention of ryegrass staggers in grazing sheep. *New Zealand Journal of Experimental Agriculture 1(1)*: 55-57. https://doi.org/10.1080/03015521.1973.10427616

Kirwan L, Connolly J, Finn JA, Brophy C, Lüscher A, Nyfeler D, Sebastià MT. 2009. Diversity-interaction modelling: estimating contributions of species identities and interactions to ecosystem function. *Ecology 90*: 2032-2038. https://doi.org/10.1890/08-1684.1

Klotz JL, Nicol AM. 2016. Ergovaline, an endophytic alkaloid. 1. Animal physiology and metabolism. *Animal Production Science 56(11)*: 1761-1774. https://doi.org/10.1071/AN14962

Latch GCM, Potter LR, Tyler BF. 1987. Incidence of endophytes in seeds from collections of *Lolium* and *Festuca* species. *Annals of Applied Biology 111(1)*: 59-64. https://doi.org/10.1111/j.1744-7348.1987.tb01433.x

Layton DL, Fletcher LR, Litherland AJ, Scannell MG, Sprosen J, Hoogendoorn CJ, Lambert MG. 2004. Effect of ergot alkaloids on liveweight gain and urine lysergol level in ewe hoggets and heifers. *Proceedings of the New Zealand Society of Animal Production 64*: 192-196.

Logan CM, Edwards GR, Kerr GA, Williams S. 2015. Ryegrass staggers and liveweight gain of ewe lambs and hoggets grazing four combinations of perennial ryegrass and strains of endophyte. *Proceedings of the New Zealand Society of Animal Production 75*: 175-178.

Munday-Finch SC. 1997. Aspects of the chemistry and toxicology of indole-diterpenoid mycotoxins involved in tremorgenic disorder of livestock. *Mycotoxin Research 13(2)*: 88. https://doi.org/10.1007/BF02945071

Popay AJ, Hume DE. 2011. Endophytes improve ryegrass persistence by controlling insects. *Pasture persistence Symposium. Grassland Research and Practice Series 15*: 149-156. https://doi.org/10.33584/rps.15.2011.3196

Spangenberg GB, Guthridge KM, Forster JW, Sawbridge TI, Ludlow EJI, Kaur J, Rochfort SJ, Rabinovich MA, Ekanayake P. 2016. *Endophytes and related methods*. (European Patent Office Patent No. WO 2011/082455).

Stewart A, Kerr G, Rowarth J, Stevens D. 2022. *Pasture and forage plants for New Zealand*. 5th ed. Dunedin, New Zealand: New Zealand Grassland Association, 139 p. https://grassland.org.nz/books.php

Thom ER, Waugh CD, Minneé EMK, Waghorn GC. 2013. Effects of novel and wild-type endophytes in perennial ryegrass on cow health and production. *New Zealand Veterinary Journal 61(2)*: 87-97. https://doi.org/10.1080/00480169.2012.715379