The impact of selected *Epichloë* endophytes on invertebrate biodiversity in New Zealand ryegrass sheep pastures

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Abstract

This study investigated the impact of six novel perennial ryegrass-*Epichloë*-associations in New Zealand sheep pasture on the above-ground and subterranean invertebrate fauna. The perennial ryegrass-endophyte treatments included cv. GA66 without endophyte, and infected with the selected *Epichloë* strains AR1, AR37 and the common-toxic endophyte. Additionally, two further perennial ryegrass lines, cv. GPD and cv. GPT, both infected with *Epichloë* strain AR37, were included to examine a cultivar effect. Invertebrates from above ground and subterranean environments were collected from pastures sown with each ryegrass-endophyte treatment at six intervals during 2021. Analysis of above-ground invertebrates found significant differences in density across ryegrass-endophyte treatments for Aranae and Opiliones, Acari, Psocoptera, Neuroptera, and *Listronotus bonariensis* (Argentine stem weevil), but overall differences for other taxa were not significant. Analysis of the Richness Index for all above-ground invertebrate taxa found significant differences amongst treatments, but not for Shannon's diversity index H. Overall, all taxa showed a decline in density following sheep grazing, but the effect was taxa specific, with Acari, Coleoptera (beetles) and Hymenoptera (wasps, bees and ants) showing the most significant impact across all ryegrass-endophyte treatments.

The most abundant soil invertebrates were Annelida (earthworms) and larvae of *Costelytra giveni* (New Zealand grass grub), with peak densities of both taxa observed in June (winter). Earthworm densities were significantly higher under all treatments following sheep grazing compared to pre-grazing densities, but the response varied for endogeic, epigeic and anecic earthworm taxa. Population fluctuations reflected grazing by sheep and the presence of dung introduced to the soil, soil moisture and season. There appeared to be minimal ryegrass-endophyte treatment effects on earthworm abundance or diversity, whereas for *C. giveni* larvae there were significant ryegrass-endophyte effects, attributed to colonisation flights in summer. Overall, large herbivore grazing had a significant impact on above-ground and subterranean invertebrate communities, irrespective of ryegrass-endophyte associations and invertebrates biology associated with seasonal changes, with the scale of impact being taxa dependent.

Keywords: alkaloids; biocontrol; earthworms, insect pests, insect-plant interactions, multitrophic interactions

Introduction

Perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) underpin New Zealand's grassland farming systems, supporting a range of pastoral industries, including dairy, beef, sheep and deer. Populations of invertebrate pests, both endemic and exotic, represent a key challenge to the productivity and persistence of cultivated ryegrass (Ferguson et al. 2019; Chapman et al. 2024). Such impacts on *Lolium* spp. can be mitigated in new pastures by utilising selected strains of asexual *Epichloë* fungal endophyte (Johnson et al. 2013). Selected strains of these *Epichloë* spp. can form mutualistic associations with perennial ryegrass, protecting plants from pest attack (Prestidge et al. 1982; Caradus 2024; Card et al. 2024). Fungal-derived secondary metabolites, most notably alkaloidal compounds, are produced as a result of this mutualistic grass-endophyte association and depending on the endophyte strain and corresponding chemical profile, these alkaloids can confer protection to both plant foliage and roots. For this reason, endophytes have been found to be crucial across many regions of New Zealand reducing the impacts of both above-ground invertebrates, such as Argentine stem weevil

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(*Listronotus bonariensis*, Kuschel), African black beetle (*Heteronychus arator* F), and porina moth species (*Wiseana* spp. Viette), along with subterranean pests, such as root aphid (*Aploneura lentisci*, Passerini) and pasture mealybug (*Balanococcus poae*, Maskell).

While endophytes can protect plants from herbivorous invertebrates, there are limited studies investigating how specific *Epichloë* strains impact other invertebrates (including above-ground) including beneficial taxa. While some studies have indicated negligible impacts of endophytes on earthworm populations (Prestidge et al. 1997; Popay & Jensen 2005), Pennell et al. (2018) found that the *Epichloë* sp. strain AR95 had a negative impact on earthworm abundance in the field after two years. In a pot trial, examining the effect of *L. perenne* with endophyte-free (E-), common-toxic (CT), AR1, and AR37 on soil nematode and microbial abundance, Bell et al. (2009) found differences in nematode functional groups and microbial communities, but the results were not consistent across different sampling dates. The effects of strains AR1 and AR37 on earthworms and non-target invertebrates under field conditions are unknown.

In this contribution, a *L. perenne*/endophyte trial was established to investigate the effect of a range of endophyte strains and *L. perenne* cultivar combinations on sheep health parameters, including effects on the gut microbiome. Endophyte strains included AR1 (known to protect grass hosts against Argentine stem weevil and pasture mealybug as well as a low level of control against black beetle), and AR37 (known to protect grass hosts against Argentine stem weevil, porina, pasture mealybug, root aphid and black beetle adults). AR37 may also reduce populations of New Zealand grass grub (*Costelytra giveni* Coca-Abia & Romero-Samper) under field conditions (Thom et al. 2014). The study presented an opportunity to determine the ecological implications of such treatment combinations on both the above-ground and subterranean populations of invertebrates.

Materials and Methods

Site details

A perennial ryegrass-endophyte experiment was established in September 2020 on a sheep pasture located on the AgResearch Lincoln research farm (S43.6324°, E172.4698°). The soil was a Wakanui imperfectly drained silt loam (mottled Immature Pallic Soil, S-Map) with two thirds of the site being Wakanui sibling 3 and a third Wakanui sibling 6. The two soil types were not distinct but merged in the lower southern third of the research site. Overall, the P-retention was 18%, the soil pH was 6.0 (0–7.5 cm depth) and soil carbon stocks 90.1 tonnes/ha (0-30 cm).

In preparation for sowing, the site was sprayed with the herbicide glyphosate (1,470 g/ha) in July 2020, then ploughed to a depth of 15-20 cm and in the following month harrowed and rolled. The top 5-10 cm was then cultivated with a power harrow in early September 2020 to create a firm fine seedbed prior to sowing. The *L. perenne* seed was sown at 25 kg/ha with a Duncan Renovator drill at 15 cm row spacing, seed was not treated with an insecticide. Six perennial ryegrass-endophyte monoculture treatments were sown in a randomised block design comprising three replicates. Each plot measured 50 m × 32.5 m and plots were irrigated as per normal practice throughout October to support germination and establishment with a total of 50 mm of irrigation applied in three passes. In early November 2020 all pastures were sprayed with a mixture of Tropotox, Versatill and Preside herbicides (1,125 g/ha MCPB, 75 g/ha MCPA, 150 g/ha clopyralid, 52 g/ha flumetsulam) for the control of broadleaf weeds. Nitrogen fertiliser in the form of urea or ammonium sulphate was applied at 40 kg N/ha in January, March, June, August, and October 2021.

Ryegrass-endophyte treatments

The six perennial ryegrass-endophyte treatments were *L. perenne* breeding line GA66 (diploid), without endophyte (E-), infected with the New Zealand common toxic strain (CT) associated with mammalian toxicity, and two commercial strains selected for their known bioactivity towards invertebrate pests; AR1, and AR37. The two other lines were GPD (diploid) and GPT (tetraploid), both infected with AR37. GDP and GPT were included to test for cultivar effects. For conciseness therefore, the line/endophyte combination treatments are hereafter referred to as GA66 E-, GA66 CT, GA66 AR1, GA66 AR37, GPD AR37, and GPT AR37.

Viable endophyte infection frequencies were determined in January (summer) and again in November (spring) 2021. On both occasions 50 ryegrass tillers were randomly selected from within each plot and the endophyte status of each tiller was assessed using an established tissue print-immunoblot technique (Simpson et al. 2012).

Animal grazing

The experiment was grazed on four occasions in 2021, these being February/March, May/June, September/October and November/December. The total number of grazing days ranged from 28-42 days at each grazing interval. The stocking rate was typically 57 sheep/ha (10 sheep/plot), however, during the first grazing period, this was augmented on a plot-by-plot basis to account for different levels of grass availability. The target was to maintain comparable grass availability across the trial area over each grazing period.

Soil moisture and irrigation

Rainfall and soil moisture deficit data were obtained from the Lincoln-Broadfield Electronic Weather Station (17603), which is located c. 1 km from the trial site. The trial was irrigated using a lateral sprinkler irrigator on the 9th of January, 19th of March and the 31st of March 2021 with 15, 20 and 20 mm of water applied per pass, respectively.

Invertebrate sampling

Sampling design

Sampling for above-ground invertebrates was carried out pre- and post-grazing (Figure 1). Due to the length of grazing intervals, the pre- and post-sampling often occurred across different seasons. Prior to the first grazing, invertebrates were sampled on the 25th of January, and then on the 17th of March, two days after grazing. Invertebrates were sampled on the 3rd of May before the second grazing and then on the 27th of June, 18 days after grazing. Invertebrates were then sampled on the 1st of November before the third grazing, and then on the 20th of December 2021, one day after grazing.

For subterranean invertebrates, an initial sample was taken on the 29th of September 2020, 6-15 days after sowing to establish baseline invertebrate densities. Subsequently, invertebrate sampling occurred prior to the first grazing on the 26th of January and then on the 18th of March 2021, three days after grazing. Invertebrates were sampled on the 4th of May before the second grazing and then on the 15th of June, six days after grazing. Invertebrates were sampled on the 3rd of November and then on the 21st of December 2021, two days after grazing.

Above-ground invertebrate sampling was generally carried out before the sampling of subterranean invertebrates, to avoid any biases that could have arisen from disturbance to the foliage. The exception to this was the June sample, where excessive moisture on the vegetation prevented the use of the sampling and was therefore postponed until the vegetation was dry 12 days after subterranean sampling, and 18 days after the sheep had been removed from the plots. The delay allowed the invertebrate population to recover from any disturbances resulting from trampling and coring around the sample points. Invertebrate sampling was not undertaken during September and October due to restrictions to staff access because of COVID-19 mandates.

Within each of the eighteen 50 m × 32.5 m plots, 10 sample points were randomly allocated using ArcGIS Pro 2.8 software (Esri Inc., USA). These points were staked-out using removable fibre-glass poles which were used as guides for each the four sampling visits, samples were taken from within <0.4 m of each point. For logistical reasons, only three of the 10 marked points within each plot were randomly selected and used for sampling above-ground invertebrates, the same three points were sampled prior to and after the sheep grazing cycle. Three points within each plot were randomly selected for each of the three grazing cycles. Conversely, all 10 points were sampled for subterranean invertebrates at each sampling date (detailed below).

Sampling above-ground invertebrates

Above ground sampling was carried out using a modified 2-stroke petrol powered blower vac (Echo ES-2100 Shred-n-Vac™, Yamabiko Corporation, Tokyo, Japan), hereafter referred to as a G-vac. The 76 cm × c.10 cm diameter intake tube was square cut at the end and a hard plastic rim fitted inside to retain a mesh 'sock' into which the sampled material was trapped. The sampled area was delineated by a metal cylinder (15 cm high × 46 cm diameter; area = 0.1666 m^{-2}) pressed into the soil, and this was vacuumed at maximum throttle for 30-90 seconds, depending on the nature of the foliage. This configuration prevented the inadvertent collection of invertebrates from the adjacent areas (Cherrill 2015). A total of 54 samples were collected on each date.

Samples collected were returned to the laboratory and sieved (5.23 mm width, 4000 µm) to remove coarse plant debris. The sorted samples were then placed in Berlese funnels where live invertebrates were extracted for 48 hours under 100w light bulbs. To prevent the escape of any flying species fine mesh lids were placed over the sample containers. Systematic specimen identification and enumeration, at least at Order level, was carried out in 8.5 cm Petri dishes and under a binocular microscope. Thereafter, the samples were stored in sealed 5 mL vials containing 70% ethanol. Acari and Collembola were often found in numbers too high to practically count and identify to higher taxonomic levels and required the recovered populations to be sub-sampled. To facilitate this, an 8.5 cm Petri dish was divided in half, the extracted sample distributed in the dish and the Acari and Collembola present on one half of the dish counted. This count was then doubled to get an estimate of the total population for both taxa. The results from the three samples per plot were then converted into the mean density m^{-2} per plot and from this the means and SEMs across the 18 plots were calculated.

Sampling subterranean invertebrates

On each of the six sampling dates from January to December, ten cores were collected using a handheld corer from within 0.4 m of each of the 10 marked areas in each plot avoiding areas that had earlier been sampled. These cores were 10 cm diameter and taken to a depth of 10-12 cm.

The vegetation on each core was removed at soil level with shears after which the cores were placed in separate plastic bags and held at 4°C prior to laboratory sorting. These were hand sorted, with the soil crumbled and the invertebrates removed, counted and identified. Soil invertebrates included earthworms and insect larvae or nymphs. For root aphid and pasture mealybug, their incidence was noted simply as presence/absence in each core. Prior to analysis, densities of each variable m^{-2} observed in 10 core counts from each plot on each sampling date were summed to calculate total count per plot.

Statistical analyses

Above-ground taxa

The total-per-plot counts for each taxon were compared between treatments on each sampling day via analysis of variance (ANOVA). ANOVA was applied to data on each sampling day separately from data on other sampling days. Each ANOVA consisted of the treatment factor for comparison and the effects of replicated plot blocks (replicate effects) for blocking. Prior to all ANOVAs, the total-per-plot counts were $\log_e(x+1)$-transformed to stabilise variation. The addition of 1 before the $\log_e$-transformation was required to include observed zero total-per-plot counts into the comparison. For some taxa, the comparisons were not made when the mean densities were very low in all treatments on a particular sampling day. Within each ANOVA, treatments were compared pair-wisely, using the least significant difference (LSD) method.

Further analysis compared overall mean values across the six sampling dates for above-ground invertebrate (1) Density; (2) Richness and (3) Shannon diversity index H; as well as above-ground Coleopteran (4) Richness and (5) Shannon diversity index H, after $\log_e(x+1)$-transformation, with the same ANOVAs. This was followed by comparison of overall mean values across the pre-grazing samples (January, May and November), with post-grazing samples (March, June and December) within each treatment for each of the variables. This pre- vs. post-grazing comparison was made on calculated pre- vs. post-grazing difference values per plot using the same ANOVA, by testing whether the treatment group's mean difference was statistically significantly different from zero.

Subterranean taxa

As with the above-ground taxa, the $\log_e(x+1)$-transformed total-per-plot counts for subterranean invertebrates were compared between treatments on each sampling date in the same way using ANOVAs. The pre- vs post-grazing differences per plot were compared in ANOVAs to see if the differences were significantly different from zero.

Of each presence rate variable, the presence per core was pooled into presence data per plot. This means, if each taxon was present in at least one soil core out of 10, that taxon was recorded as present in the plot. Conversely, taxon was recorded as absent in a plot if that taxon was not found in any of the 10 cores. These presence-per-plot data were compared between treatments within each sampling date, using a generalised linear model (GLM) with binomial distributions through a logit link function. Each GLM analysis consisted of treatment factor and replicate blocking effects. All ANOVAs were carried out with the statistical software package SAS version 9.4 (SAS Institute Inc., USA), whereas GLM analyses were undertaken with Minitab Statistical Software Version 16.2.2 (Minitab 2021).

Results

Soil moisture and irrigation

Between the 1st of December 2020, and up to the first sampling date on the 26th of January 2021, c. 66 mm of rain was recorded, with 15 mm of irrigated water applied on the 9th of January (Figure 1). Between the 28th of January and the 19th of March c. 39 mm of rain fell. Soil moisture deficits were relatively high during this period (Figure 1). From the 19th of March to the 6th of May, a total of 38 mm of rain was recorded, with a further 20 mm of water applied as irrigation on the 19th of March and again on the 31st of March (total 40 mm). Soil moisture deficits showed a steep decline after the 25th of May. From the 6th of May to the 16th of June, 157 mm of rainfall was recorded, and between the 17th of June and the 4th of November, c. 227 mm of rain was recorded. Approximately 130 mm of rain was recorded between the 5th of November and the 21st of December 2021.

Endophyte infection frequencies

Viable endophyte infection frequencies in January ranged from 1 to 97% (Table 1). The subsequent assessment in November 2021 recorded similar endophyte infection frequencies for the GA66 lines

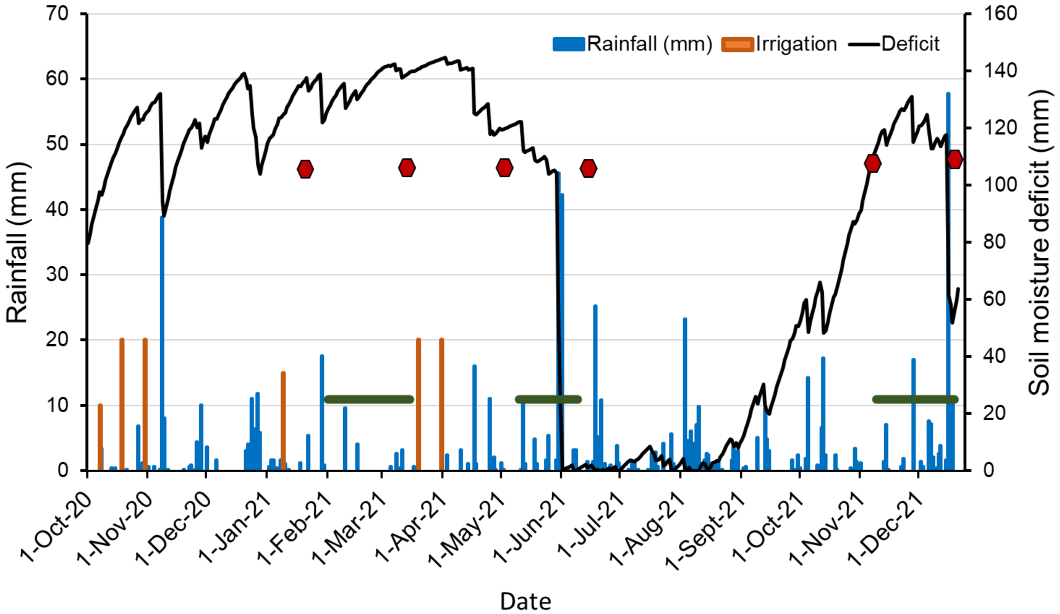


Figure 1 Rainfall (mm), irrigation and soil moisture deficit (mm) data recorded during the ryegrass-endophyte trial. Rainfall and soil moisture deficit data obtained from the Lincoln-Broadfield Electronic Weather Station (17603). Sampling dates are indicted by red hexagons while the grazing intervals are indicated by green lines.

(Table 1). Endophyte infection frequencies were not assessed for GPD AR37 and GPT AR37.

Ryegrass-endophyte effects on above-ground invertebrates

The above-ground taxa was comprised largely of Aranae and Opiliones (spiders and harvestmen, respectively), Acari (mites), Myriapoda (millipedes and centipedes), Coleoptera (beetles and weevils, both larvae and adults), Hymenoptera (parasitoids and ants), Neuroptera (lacewings, both larvae and adults), Thysanoptera (thrips), Pscoptera (barklice), Hemiptera (plant bugs), Sminthuridae (globular springtails), Collembola (springtails), Diptera (flies, both larvae and adults), Lepidoptera (moths, both larvae and adults) and *L. bonariensis* (adults).

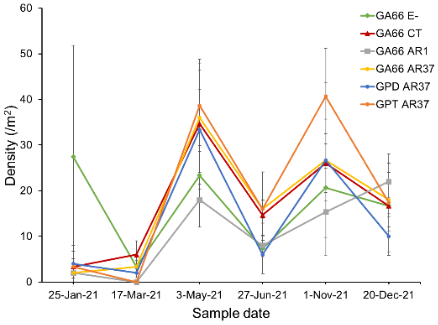
Changes in the densities of selected taxa on the six sampling dates and each ryegrass-endophyte treatment are presented in Figure 2. Due to the low incidence and densities, results for Myriapoda (millipedes, centipedes) and *Nysius huttoni* (wheat bug) are not presented.

When the sampling dates were combined, there were some significant (P<0.05) treatment differences between the invertebrate taxa (Table 2). However, the high levels of catch variation reduced the levels of significance overall. The densities of Aranae and Opiliones were greatest within plots of GPT AR37 (19.3 m^{-2}), with significantly lower densities recorded for plots of GA66 AR1 (10.9 m^{-2}) which was significantly less than the GPT AR37 treatment (Table 2). Similarly, the lowest densities of Acari were within the plots of GA66 AR1 (584 m^{-2}), which were significantly less than within plots of GA66 CT, GA66 E-, GPD AR37, and GPT AR37. Adult densities of *L. bonariensis* were lowest in plots of GA66 AR1 (1.6 m^{-2}), and significantly lower than GA66 E- and GPT AR37 (3.6 m^{-2}). Dipteran densities were greatest in plots with GA66 AR37 (26.2 m^{-2}), being significantly greater than

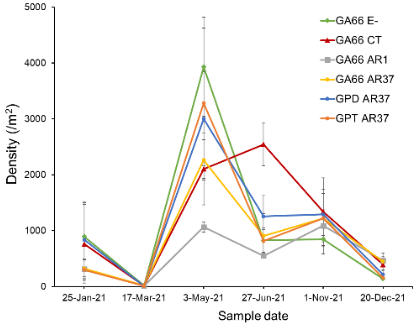
Table 1 Endophyte infection frequencies in the line-endophyte combinations for January and November 2021. Viable endophyte infection frequencies were not measured assessed for GPD AR37 and GPT AR37 in November.

Treatment	January	November
GA66 E-	1	2
GA66 CT	97	90
GA66 AR1	95	97
GA66 AR37	95	91
GPD AR37	97	N/A
GPT AR37	80	N/A

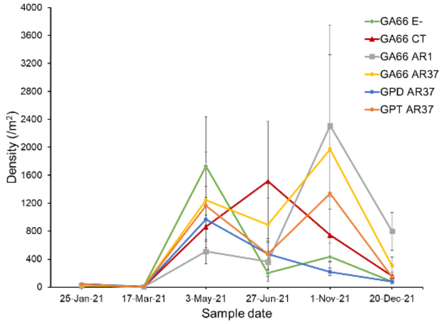
A) Aranae and Opiliones



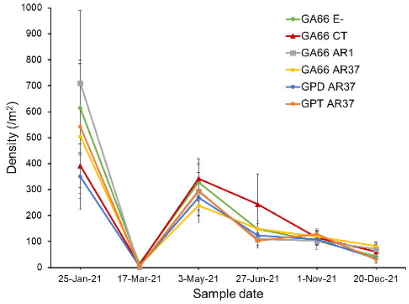
B) Acari



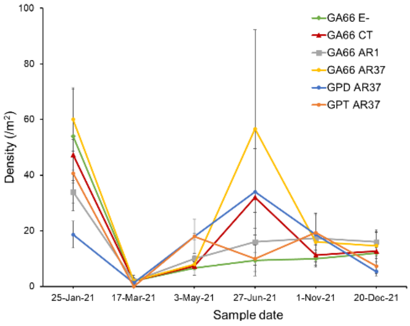
C) Collembola



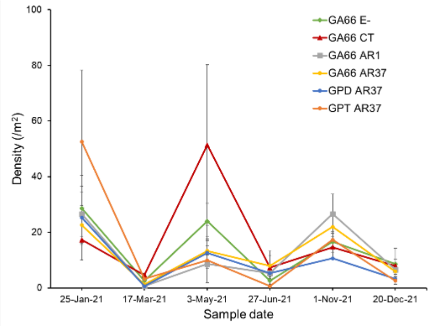
D) Coleoptera



E) Diptera



F) Hymenopteran



G) *Listronotus bonariensis*

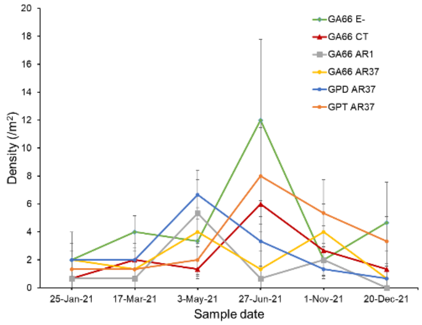


Figure 2 Mean density (m^{-2} ± SEM) changes for A) Aranae and Opiliones (spiders and harvestmen), B) Acari (mites), C) Collembola (springtails), D) Coleopteran taxa (larvae and adults), E) Dipteran (larvae and adults), F) Hymenopteran (wasps, parasitoids, ants) taxa and G) *L. bonariensis.* Samples collected from the ryegrass-endophyte treatments (GA66 E-, GA66 CT, GA66 AR1, GA66 AR37, GPD AR37, GPT AR37) pre- (25th of January, 3rd of May, 1st of November 2021) and post-grazing (17th of March, 27th of June, 20th of December 2021) by sheep.

all treatments with the exception of GA66 CT (18.8 m^{-2}). Densities of Psocoptera on plots with GA66 E- were significantly greater compared with plots with GA66 AR37, GA66 AR1 and GPD AR37. Neuroptera (lacewings) densities were low across all treatments (0 to 0.6 m^{-2}) but were greatest on plots with GA66 E- (0.6 m^{-2}), which was significantly different to densities recorded on plots with GA66 AR37 (0.0 m^{-2}, $P=0.034$). No significant treatment effects were found for Collembola, Coleoptera, Hymenoptera, Thysanoptera, Sminthuridae, Hemiptera and Lepidoptera (data not shown). There were significant block effects (three blocks) for Aranae and Opiliones ($P<0.001$), Acari ($P=0.004$) and Diptera ($P<0.001$) densities (Table 2). For all three taxa, block 3 supported significantly larger populations than block 1.

All invertebrate taxa combined for all sampling dates, showed that plots with GA66 E- had the highest

Table 2 Mean density (m^{-2}) (± SEM) of selected above-ground invertebrate taxa across all sampling dates (25th of January, 17th of March, 3rd of May, 27th of June, 1st of November 2021 and 20th of December 2021) for the ryegrass-endophyte treatments. For each variable, treatments that share a common letter do not differ statistically significantly at 5% significance level. Significance of replicate block effects indicated by the P-value with those < 0.05 in bold.

Treatment	**Aranae and Opiliones**	**Acari**	**Psocoptera (barklice)**	**Neuroptera (lacewings)**	**Diptera**	***L. bonariensis***
GA66 E-	16.5 ± 6.62 **ab**	1110 ± 181 **a**	7.2 ± 1.79 **a**	0.6 ± 0.40 **a**	15.7 ± 2.50 **b**	4.7 ± 0.67 **a**
GA66 CT	16.9 ± 3.30 **ab**	1192 ± 324 **a**	4.3 ± 0.19 **ab**	0.1 ± 0.11 **ab**	18.8 ± 1.44 **ab**	2.3 ± 0.38 **bc**
GA66 AR1	10.9 ± 1.79 **b**	584 ± 51 **b**	2.2 ± 0.89 **b**	0.1 ± 0.11 **ab**	15.9 ± 2.52 **b**	1.6 ± 0.78 **c**
GA66 AR37	17.0 ± 5.92 **ab**	866 ± 154 **ab**	2.6 ± 0.87 **b**	0.0	26.2 ± 7.06 **a**	2.2 ± 0.29 **bc**
GPD AR37	13.7 ± 4.18 **ab**	1102 ± 111 **a**	1.6 ± 0.91 **b**	0.2 ± 0.11 a**b**	16.0 ± 2.80 **b**	2.7 ± 0.19 **abc**
GPT AR37	19. 3 ± 3.72 **a**	966 ± 280 **a**	3.3 ± 0.96 **ab**	0.3 ± 0.19 **ab**	15.9 ± 3.30 **b**	3.6 ± 0.73 **ab**
Block effect	**P < 0.001**	**P = 0.004**	P = 0.858	P = 0.409	**P<0.001**	P=0.499

Table 3 Mean (± SEM) Richness and Shannon's diversity index H calculated for above-ground invertebrate taxa and coleopteran taxa across all sampling dates (25th of January, 17th of March, 3rd of May, 27th of June, 1st of November 2021 and 20th of December 2021) for the ryegrass-endophyte treatments. For each diversity index, treatments that share a common letter do not differ statistically significantly at 5% significance level. Significance of replicate block effects indicated by the P-value with those < 0.05 in bold.

Above-ground invertebrates		
Treatment	**Richness**	**Shannon's diversity index H**
GA66 E-	10.11 ± 0.53 **a**	1.248 ± 0.013 **a**
GA66 CT	9.52 ± 0.23 **ab**	1.145 ± 0.072 **a**
GA66 AR1	8.91 ± 0.25 **b**	1.142 ± 0.049 **a**
GA66 AR37	8.91 ± 0.51 **b**	1.114 ± 0.061 **a**
GPD AR37	9.24 ± 0.35 **ab**	1.098 ± 0.036 **a**
GPT AR37	8.98 ± 0.52 **b**	1.115 ± 0.041 **a**
Block effect	P=0.079	P=0.840
Above-ground Coleoptera only		
Treatment	**Richness**	**Shannon's diversity index H**
GA66 E-	4.13 ± 0.13 **a**	0.945 ± 0.040 **a**
GA66 CT	4.04 ± 0.13 **a**	0.791 ± 0.030 **c**
GA66 AR1	3.91 ± 0.13 **a**	0.793 ± 0.027 **c**
GA66 AR37	4.00 ± 0.13 **a**	0.773 ± 0.033 **c**
GPD AR37	4.04 ± 0.13 **a**	0.888 ± 0.030 **ab**
GPT AR37	3.82 ± 0.13 **a**	0.844 ± 0.049 **bc**
Block effect	P=0.424	P=0.840

richness and both GA66 AR1 and GA66 AR37 the lowest (Table 3). There were also significant differences of GA66 E- from GA66 AR1 and GA66 AR37 (both P=0.037), and between GA66 E- and GPT AR37 (P=0.048). Shannon's diversity index H was highest in plots with GA66 E- and lowest in plots with GPD AR37, but there were no significant differences across ryegrass-endophyte treatments (Table 3).

Shannon's diversity index H was significantly greater in plots with GA66 E- followed by GPD AR37. The diversity index H was lowest for the grouping of GA66 AR37, GA66 CT and GA66 AR1 treatments (Table 3). The diversity index was significantly different between plots with GA66 E- and GA66 CT (P=0.002), GA66 AR1 (P=0.002), GA66 AR37 (P<0.001), and GPT AR37 (P=0.021), respectively. Significant differences were also found within plots of GPD AR37 from GA66 AR1 (P=0.027), GA66 AR37 (P=0.011), and GA66 CT (P=0.024), respectively.

Pre- and post-grazing impacts on above-ground invertebrates

Overall, all taxa showed density declines following grazing. For Coleoptera this was statistically significant across all ryegrass-endophyte treatments, and for Hymenoptera all treatments except for GPD AR37 (Table 4). Significant declines were found for the Aranae and Opiliones, and Acari in the GA66 E-, GPD AR37 and GPT AR37 treatments, and Acari only for GA66 AR37 (Table 4). Conversely, grazing had no statistically significant impact on population densities of Collembola, while for Diptera, Thysanoptera, Sminthuridae, Hemiptera, and Lepidoptera the impact of grazing was generally non-significant within taxa, apart for significant differences in only one of the ryegrass-endophyte treatments, mainly associated with GPD AR37 and GPD AR37. Grazing did not appear to impact *L. bonariensis* adults, with the only significant difference observed in GA66 E- (P=0.010), where there was an overall significant increase in density (Table 5). This was attributed to adult recruitment in autumn (May) (2nd adult generation) and again in November (spring) and December (early summer) (1st adult generation) (e.g., Goldson et al. 2011). Significant block

Table 4 Mean density (± SEM) of Aranae and Opiliones, Acari, Coleoptera and Hymenoptera for all pre- (25th of January, 3rd of May, 1st of November 2021) and post- grazing (17th of March, 27th of June, 20th of December 2021) sampling dates for the ryegrass-endophyte treatments. P values are shown for pre- and post- grazing density differences and block effect, with those < 0.05 are in bold.

	Aranae and Opiliones			**Acari**		
Treatment	**Pre-graze**	**Post-graze**	**P values**	**Pre-graze**	**Post-graze**	**P values**
GA66 E-	23.79 ± 11.12	9.12 ± 2.18	**P=0.012**	1891 ± 304	329 ± 74	**P<0.001**
GA66 CT	21.35 ± 6.36	12.45 ± 1.18	P=0.092	1401 ± 528	984 ± 142	P=0.167
GA66 AR1	11.78 ± 2.92	10.00 ± 0.67	P=0.717	824 ± 85	344 ± 22	P=0.117
GA66 AR37	21.57 ± 7.40	12.44 ± 4.52	P=0.085	1272 ± 126	460 ± 187	**P=0.016**
GPD AR37	21.35 ± 8.36	6.00 ± 0.0	P=0.009	1711 ± 300	493 ± 117	**P<0.001**
GPT AR37	27.57 ± 5.00	11.12 ± 3.14	**P=0.006**	1603 ± 540	329 ± 65	**P<0.001**
Block effect	P=0.232			P=0.058		
	Coleoptera			**Hymenoptera**		
Treatment	Pre-graze	Post-graze		Pre-graze	Post-graze	
GA66 E-	348.14 ± 91.44	68.47 ± 3.28	**P=0.003**	23.13 ± 3.74	4.66 ± 2.70	**P=0.012**
GA66 CT	282.91 ± 57.42	104.64 ± 33.01	**P=0.030**	27.79 ± 10.62	6.67 ± 1.39	**P=0.006**
GA66 AR1	368.35 ± 73.63	60.68 ± 16.43	**P<0.001**	20.67 ± 2.52	4.22 ± 1.11	**P=0.021**
GA66 AR37	286.91 ± 112.85	80.03 ± 10.34	**P=0.015**	19.34 ± 1.76	5.12 ± 2.12	**P=0.039**
GPD AR37	242.76 ± 8.70	54.68 ± 10.34	**P=0.024**	16.23 ± 6.18	3.11 ± 1.24	P=0.054
GPT AR37	322.73 ± 119.25	45.58 ± 11.82	**P=0.003**	26.67 ± 7.20	2.22 ± 0.22	**P=0.002**
Block effect	**P=0.037**			P=0.113		

Table 5 Mean (± SEM) density of *L. bonariensis* for all pre- (25th of January, 3rd of May, 1st of November 2021) and post- grazing (17th of March, 27th of June, 20th of December 2021) sampling dates for the ryegrass-endophyte treatments. P values are shown for pre- and post- grazing density differences and block effect and those < 0.05 in bold.

	L. bonariensis		
Treatment	Pre-graze	Post-graze	P-value
GA66 E-	2.44 ± 0.44	6.89 ± 1.60	**P=0.010**
GA66 CT	1.56 ± 0.59	3.11 ± 1.24	P=0.291
GA66 AR1	2.67 ± 1.15	0.44 ± 0.44	P=0.143
GA66 AR37	3.34 ± 0.67	1.11 ± 0.22	P=0.143
GPD AR37	3.34 ± 0.38	2.00 ± 0.67	P=0.362
GPT AR37	2.89 ± 0.44	4.22 ± 1.35	P=0.362
Block effect	P=0.508		

effects were only found for Coleoptera (P=0.037), with numbers significantly higher in block 2, compared to blocks 1 and 3.

Subterranean invertebrates

Initial sampling 6-15 days after sowing found the incidence and density of subterranean taxa in plots was negligible, with only two *C. giveni* larvae and 12 earthworms recovered in total across the 18 plots. The two *C. giveni* larvae were recovered from separate GA66 AR37 plots, while only low densities of earthworms (≤15 m^{-2}) were recorded across the five ryegrass-endophyte treatments.

Overall, across the January to December 2021 sampling, the most abundant invertebrates found were earthworms and *C. giveni* larvae, followed by root aphid and pasture mealybug, although only presence and absence were recorded for the latter two species. Other insects that were infrequently found in the soil included Porina sp. (a total of 12 caterpillars), Tasmanian grass grub (*Acrossidius tasmaniae* Hope) (31 larvae), wireworm (Coleoptera: Elateridae) (11 larvae) and fly larvae (2).

Earthworms

There appeared to be no obvious effect of ryegrass-endophyte treatment on earthworm abundance throughout the year (Figure 3). Earthworm densities were initially low (17 weeks after sowing) at the commencement of sampling in January and remained low through to the 19th of March (Figure 3). Thereafter, densities increased in the May sampling, through to the 16th of June, followed by a decline on the 4th of November. The final sampling on the 21st of December showed a jump in densities across all treatments (Figure 3). Within sample dates, significant treatment effects were only observed in the January sample, in part because relatively more earthworms were observed in some plots of the GA66 CT and GA66 AR37 treatments.

The predominant earthworm species found were endogeic *Aporrectodea caliginosa,* other endogeic species included *Octolasion cyaneum, Aporrectodea rosea, Aporrectodea trapezoides*. Epigeic *Lumbricus rubellus* and anecic *Aporrectodea longa* were also observed but not influenced by treatment. Significant replicate block effects were only seen for the 26th of January (P=0.050) and the 21st of December (P=0.026) samples. This was due to significantly lower mean density in block 3 compared to compared to blocks 1 and 2.

Earthworm densities were significantly greater under all treatments following the May-June and November-December grazing periods compared to pre-grazing densities (Table 6). The change was particularly apparent for GPD AR37 and GA66 AR1, with densities 8.85 and 5.04 times higher post-grazing, respectively (Table 6), These periods of grazing also coincided with increased rainfall and hence soil moisture. Between the 6th of May and the 16th of June, 157 mm of rainfall was recorded, while between the 17th of June and the 4th of November, c. 227 mm of rain fell. Approximately 131 mm of rain was recorded between the 5th of November and the 21st of December 2021, with 75 mm recorded from the 16th to the 18th of December alone, which represented 57% of the total rainfall measured over that period. Densities of endogeic and anecic species peaked in winter (Figure 4) but showed a significant decline when sampled on the 3rd of November. Endogeic densities increased again on the 21st of December sample, while anecic taxa continued to decline. Densities of epigeic taxa were low for most sampling occasions but did show an increase in the December sampling. Increased earthworm abundance largely reflected increasing endogeic immature earthworms (Figure 4).

Costelytra giveni larval densities

Costelytra giveni larval densities showed an increase

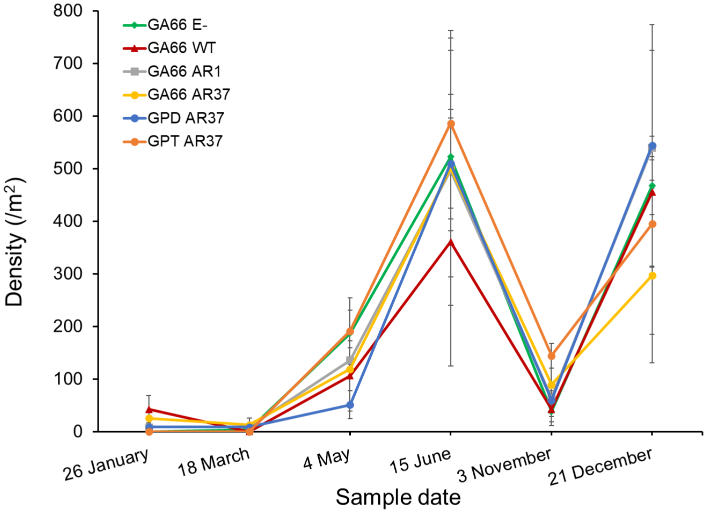


Figure 3 Mean earthworm densities (m^{-2} ± SEM) measured under the ryegrass-endophyte treatments (GA66 E-, GA66 CT, GA66 AR1, GA66 AR37, GPD AR37, and GPT AR37) pre- (25th of January, 3rd of May, 1st of November 2021) and post- grazing (17th of March, 27th of June, 20th of December 2021).

Table 6 Mean (±SEM) earthworm densities (/m^2) pre- (25th of January, 3rd of May, 1st of November 2021) and post-grazing (17th of March, 27th of June, 20th of December 2021) for the combined ryegrass-endophyte treatments. The P-value indicates the level of significance between the two grazing events.

Treatment	Pre-grazing time	Post-grazing time	P-value
GA66 E-	75.03 ± 9.29	331.24 ± 46.76	**0.005**
GA66 CT	63.70 ± 39.49	271.36 ± 168.17	**0.015**
GA66 AR1	69.36 ± 6.17	348.18 ± 31.85	**0.003**
GA66 AR37	77.84 ± 39.11	270.09 ± 140.14	**0.022**
GPD AR37	39.62 ± 14.78	354.17 ± 150.33	**0.001**
GPT AR37	111.86 ± 27.90	327.04 ± 78.86	**0.012**

across all ryegrass-endophyte treatments through to the June sampling, although the change in respective densities did vary with treatment (Figure 5). On the 3rd of November, only seven larvae were recorded across all GPD AR37 and GA66 AR1 treatments, with none from other treatments. By comparison, a total of two pupae and 40 adults were recorded across all plots, and therefore not included in the analysis. Next generation, first instar larvae were recorded at low densities in December.

With regard to the peak larval densities in June, GA66 AR1 supported significantly higher densities than both GA66 E- (P=0.001) and GPD AR37 (P=0.018). Conversely, densities in GA66 E- were significantly less than GPT AR37 (P=0.024), GA66 AR37 (P=0.013) and GA66 CT (P=0.042).

Prevalence of root aphid (*Aploneura lentisci*) and pasture mealybug (*Balanococcus poae*)

Neither root aphid nor pasture mealybug were detected in the January and March samples but occurred in the four subsequent sampling events. There was no ryegrass-endophyte treatment effect. Overall, the percentage presence of root aphid across all ryegrass-endophyte treatments was highest in the 3rd of November sample, whereas percentage presence of pasture mealybug was highest in the 15th of June sample. The presence of both species was highest in the GA66 E- and/or GA66 AR1 treatments (although this was not significant). By comparison, GA66 AR37, GPD AR37 and GPT AR37 treatments had either no or a low presence rate of either species. The incidence of root aphid in the GA66 CT treatment was intermediate to GA66 E- and GA66 AR1 and the GA66 AR37, GPD AR37 and GPT AR37 treatments. By comparison, the overall prevalence of pasture mealybug under GA66 CT was low, being present only on the 4th of May and 15th of June samples.

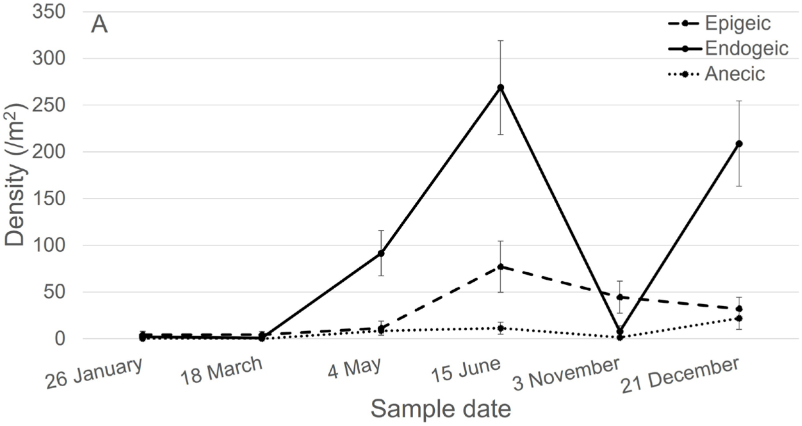


Figure 4 Mean earthworm densities (m⁻² ± SEM) for epigeic, endogeic, and anecic taxa (A) and proportion of mature, immature and juvenile stages (B), found under the ryegrass-endophyte treatments (GA66 E-, GA66 CT, GA66 AR1, GA66 AR37, GPD AR37, and GPT AR37) pre- (26th of January, 4th of May, 3rd of November 2021) and post- sheep grazing (18th of March, 15th of June, 21st of December 2021).

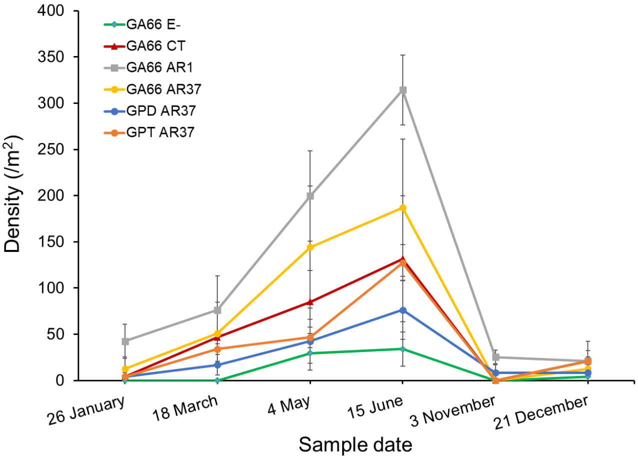


Figure 5 Mean density (m⁻² ± SEM) changes for *C. giveni* larvae collected from the ryegrass-endophyte treatments (GA66 E-, GA66 CT, GA66 AR1, GA66 AR37, GPD AR37, and GPT AR37) pre- (26th of January, 4th of May, 3rd of November 2021) and post-sheep grazing (18th of March, 15th of June, 21st of December 2021).

There were no significant replicate block effects.

Discussion

This contribution showed that in the first year following pasture establishment, the effect of ryegrass-endophyte combinations on above-ground and subterranean invertebrate densities and community diversity was taxon-specific and varied through time. Overall, the ryegrass-endophyte treatments had some significant effects on both above-ground and subterranean invertebrates irrespective of whether they are categorised as beneficial organisms or as pests.

Throughout the trial, GA66 AR1 had lower abundances of adult *L. bonariensis* in comparison to the GA66 E-, but also lower abundances of Acari, and higher abundance of *C. giveni*. *Listronotus bonariensis* remained significantly lower on GA66 AR37, but differences were not significant with other cultivars. Although there were some significant ryegrass-endophyte treatments effects, the Richness and Shannon's Diversity Index H for both total above-ground invertebrates and Coleoptera was relatively uniform across treatments. This is not surprising, as the trial consisted of a ryegrass monoculture, and plant diversity has been shown to be a key factor influencing invertebrate diversity (Zhu et al. 2012; Joern & Laws 2013; van Klink et al. 2015).

Populations of *C. giveni* showed ryegrass-endophyte effects in June when populations were likely at damaging levels (≥ 150 larvae m^{-2}) (Ferguson et al. 2019), with densities under the GA66 AR1 and GA66 AR37 treatments higher compared to the other treatments. As AR1 had no activity against larvae, and AR37 has been shown to provide a low level of control (Popay & Thom 2009; Tozer et al. 2017; Zydenbos et al. 2011), the result is perhaps not unexpected, although is in contrast to what was observed for *L. bonariensis*. *Costelytra giveni* larvae were found at low densities in December, these being the new generation arising from the spring oviposition (Fenmore 1984). However, the observed increased density from January to June was an artifact due to improved detection of larger larvae as they moved from 1st to 3rd instars. The beetle generally has a one year life cycle in Canterbury, so the same population was being resampled across the four dates (McNeill et al. 2023). *Costelytra giveni* are expected to increase in the first few years following pasture establishment and future research could consider the longer-term impacts of these ryegrass-endophyte treatments. The cause of significantly higher *C. giveni* larval populations observed in GA66 AR1 and GA66 AR37 treatments is not easily explained but may be related to the pattern of migration of adults from outside the trial site in spring and early summer, or better larval establishment under GA66 AR1 and GA66 AR37 compared to the other treatments. Another possibility could be related to the plant's spectral signature that influences adult orientation during the mating and oviposition flights. European cockchafer, *Melolontha melolontha* (L.), an agricultural pest in Europe, has been shown to orient to adjacent fields for oviposition, possibly using the skylight polarisation pattern as a cue (Labhart et al. 1992; Hegedüs et al. 2006). The adult can detect polarised light in the green range of the colour spectrum (~ 520 nm) (Labhart et al. 1992), which is associated with a crepuscular activity, where having green-sensitive compound eyes (rather than higher UV-sensitivity) is beneficial during lower solar elevations. Ryegrass-endophyte spectral signatures have been shown to differ amongst cultivars during the time *C. giveni* adults are flying, especially in the blue, green and red spectrums (P. Plentokov, AgResearch, unpublished data). Speculation is that certain signatures may act as an orientation cue for adults but obviously requires validation. Populations of *C. giveni* did not show any response to grazing.

Both root aphid and pasture mealybug feed on the roots of ryegrass, with pasture mealybug generally occurring 1-2 cm below the surface and near the crown (Charles *et al*. 2009). There was a trend showing an endophyte effect on the presence of both species, albeit at low densities, although this was not significant. This is not unexpected given the site was fully cultivated prior to sowing, thereby disrupting any existing populations, and measurements covered a time period four to 15 months following sowing. Both taxa were generally less frequently found in ryegrasses hosting AR37. Pennell et al. (2005), likewise showed an AR37 endophyte effect on reducing both root aphid and pasture mealybug feed populations in two-year pastures. Further, these workers found neither AR1 nor CT reduced root aphid numbers (Pennel et al. 2005). Both Hume et al. (2007) and Popay & Gerard (2007) found that AR37 strongly supressed root aphid numbers on GA66 roots compared to the same cultivar without endophyte, CT or AR1 endophytes. Field trials on susceptible ryegrass-endophyte cultivars have shown significant dry matter yield losses caused by both root aphid (Hume et al. 2007) and pasture mealybug (Pennell *et al*. 2005).

There was no indication of ryegrass-endophyte treatments affecting the abundance or diversity of earthworms across all treatments, which is consistent with previous research (Prestidge et al. 1997; Popay & Jensen 2005). However, this study extended these studies to include AR1 and AR37. There was a general increase in earthworm abundance throughout the trial period, with populations increasing after grazing and coinciding with increased soil moisture during the wetter months. Soil moisture is an important determinant to earthworm abundance, with a negative correlation shown between soil moisture and earthworm densities (Schon et al. 2017). The observed population increases after grazing may have also been positively affected by the accumulation of dung on the soil surface and consequent colonisation of dung feeding by the epigenic species *L. rubellus*. This species nearly doubled in abundance during this study but notably this did not apply to the endogeic species. As the amount of dung deposited during grazing was not recorded, the quantitative effect on earthworm population changes cannot be determined. Typically, earthworm abundance was expected to be highest during winter/spring, but abundance was also high in December following significant rainfall.

It was apparent that sheep grazing also had a major effect on densities of above-ground invertebrates, the extent to which was again taxon dependent. This result was to be anticipated as the effect of large herbivore pasture grazing on invertebrate densities and diversity has generally been shown to be negative, influenced by sward composition, grazing intensity and its frequency (Kruess & Tscharntke 2002; Zhu et al. 2012; Takagi & Miyashita 2014; van Klink et al. 2015). These include mortality caused by trampling, ingestion and disturbances. To this effect, large herbivores can be used in controlling herbivorous pests at critical times of the lifecycle (East & Pottinger 1983). In particular, grazing is generally found to be detrimental to beneficial groups such as predators and parasitoids (Kruess & Tscharntke 2002; Ma et al. 2017). Indeed, in this study both Coleoptera and Hymenoptera showed a significant decline in numbers following grazing, with many of these taxa classified as natural enemies (Anderson et al. 2011; Lövei & Sunderland 1996). In this study it was also shown that invertebrate populations showed local recolonisation following grazing, with some of the relative changes in numbers attributable to phenology and seasonality of specific species. This was particularly so for the May (autumn) versus June sample (winter), when densities of some taxa could be expected to be lower in winter.

Conclusion

This large pasture field study under New Zealand sheep grazing regimes has elucidated and measured invertebrate population responses to cultivar and endophyte strains both above and below the soil surface. This work supports results from controlled studies, highlighting the value of endophytes in reducing some invertebrate pasture pests. While not impacting earthworm densities, this study did show the impact of endophytes on some non-target organisms. Factors such as timing of sample collection and grazing appear to have even greater impacts on the above-ground and subterranean invertebrates and should be considered in longitudinal field research.

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