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Dry matter yield and the prevalence of barley yellow dwarf and ryegrass mosaic viruses in old and young perennial ryegrass

M.B. FARQUHAR[1], C.S. WINEFIELD[1] and C.C. EADY[2]
[1] *Lincoln University, Wine and Food Molecular Biosciences, Ellesmere Junction Rd, Lincoln 7647, New Zealand*
[2] *Agriseeds Ltd, 2547 Old West Coast Road, RD1, Christchurch, 7671, New Zealand*
ceady@agriseeds.co.nz

Abstract
Modern pasture management of perennial ryegrass results in reduced reseeding and increased reliance on asexual tiller multiplication. This may exacerbate viral impact by providing longer-living hosts to exploit, thus the effect of ryegrass age on sward performance and viral load was investigated. Genetically similar 10 year old field plants and 10 year old seed were used to produce 'mini-swards' of 'old' (tiller derived) and 'young' (seed derived) ryegrass lines. Dry matter yield and viral load (ryegrass mosaic, and barley yellow dwarf) were assessed over 10 months. For all lines the old mini-swards produced less biomass (4-29%) and viral load was significantly greater at most time points. Cause and effect between viral load and yield were not proven as other factors such as genetic drift, epigenetics, or other latent pests or diseases could not be ruled out.

Keywords: *Lolium perenne*, barley yellow dwarf virus, ryegrass mosaic virus

Introduction
At the core of the livestock industry in New Zealand is pasture (Lee *et al.* 2012), the productivity and profitability of which is inextricably linked to its quality and performance (Minneé *et al.* 2010). In New Zealand, perennial ryegrass is the most commonly used pasture grass species (Lee *et al.* 2012; Stewart *et al.* 2014). Several viruses e.g. barley yellow dwarf virus (BYDV) and ryegrass mosaic virus (RGMV) can infect ryegrass and cause economic loss (Wilkins & Catherall 1977; Latch 1980; Coutts & Jones 2002), with RGMV infection rates of up to 60% (Webster *et al*. 1996) and yield losses of up to 50% in experimental inoculated swards of Italian ryegrass (Wilkins & Hide 1976; Eagling *et al.* 1992). With New Zealand dairy farms producing ~14 t DM/ha/year (http://www.siddc.org.nz/sthld-demo-farm/farm-walk-notes/pasture-growth/) it follows that this equates to a potential worst case loss of ~4.2 t DM/ha/year or between ~$627 to $960/ha, according to the Forage Value Index (FVI,www.dairynz.co.nz) seasonal values (2016). In addition, multiple viral infections in ryegrass may act synergistically (Eagling *et al.* 1992; Guy 2014) as documented in other monocotyledons (Carfrune *et al.* 2006). The massive shift to dairying and irrigation in the South Island, and making silage instead of hay reduces natural reseeding, potentially requiring plants (tiller derived clones) to live longer and so giving viruses a greater opportunity to multiply and increase their burden on the plant. While reports on spread and incidence of viruses within individual plants in a sward are common (e.g. Webster *et al.*1996) methods to measure viral load within a sward *per se* and the impact of this, have been lacking. Yet it is the sward as a whole that the farmer is interested in; a new look at the effect of viral load in the sward on pasture performance is overdue. The hypothesis for this research is that viral load (not percentage of plants infected) within a sward is increased over time and reduces ryegrass yield and persistence.

Methods
Plant material
Five lines from part of a 10-year-old breeder's 'without endophyte' persistency trial were chosen in October 2014 from the New Zealand Agriseeds Ltd. breeding station at Courtenay, near Christchurch. These were diploid perennial ryegrasses coded Lp258, Lp256, R164, R141 and a tetraploid line called 'Bealey'. Twenty four 'mushroom-type fairy ring' ryegrass clumps (i.e. the centre had died out and the grass plant had spread by tillers growing outwards in a radial manner) per line were selected, dug up and 60 washed tillers, ~2-3 from each of the 24 plants, were mixed and transplanted into compost in germination trays (37 x 23 cm) creating 'old swards'. In parallel, stored seed from the original seed batches used for the above, were sown to create equivalent density 'young swards'. Trays of both swards were kept outside at Courtney for 2 months before transplanting in May 2015 (two trays/sward) forming spaced mini-swards surrounded by turf grass. Swards were arranged in 5 by 6 rows with each line and treatment (old or young) randomised and represented in triplicate. Swards were left for 1 month to establish (Figures 1a, 1b).

Sampling protocol
To obtain samples for yield analysis, a reel mower with the blade at the same height for each sward (~3 cm)

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was used to harvest the grass (a lower height was used initially to cut the surrounding turf grass). Samples were weighed to determine freshweight, and oven-dried at 65°C for 48 hours to determine dry matter (DM) content. The mower was not cleaned between mini-swards as measurements (virus and yield) were quantitative and contamination between samples was considered to be minimal. Spread of virus through cutting was considered part of the 'natural' process that might occur through grazing or mowing for hay and silage or 'topping' (key to this experiment was not the presence of virus *per se* but rather the build up of viral load over time). Surrounding turf grass sections were cut and sampled to determine the variability of the block. Samples for viral detection were collected at the start, middle and end of the experiment, as described above, placed on ice and stored within 2 hours of harvest at -80°C.

Analysis of yield and persistence

Yield (DM) was measured 6 times throughout the year for each sward, three at the start of the experiment going into winter and three at the end in summer going into drought. Persistence was measured as the stability of yield over time.

Virus detection and quantification

To achieve a representative subsample, frozen ryegrass samples were ground to a fine powder in liquid nitrogen and a ~1.0 g subsample stored at -80ºC. Ribonucleic acid (RNA) was isolated from 0.1 g of this tissue using a Spectrum™ Plant Total RNA Kit, in accordance with the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). RNA was treated with Ambion® Turbo DNase (ThermoFisher, Scientific, Waltham, MA, USA) to remove DNA. The concentration and purity of total RNA was assessed with a NanoDrop at 260 nm/280 nm and 320 nm according to the manufacturer's instructions (ThermoFisher, Scientific, Waltham, MA, USA) and by gel electrophoresis according to MIQE (Minimum Information for Publication of Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Experiments) guidelines (Taylor *et al.* 2010). Following this a TaKaRa BluePrint™ RT-PCR cDNA Synthesis Kit (Takara Bio, Inc., Otsu, Shiga, Japan) was used for reverse transcription of the RNA according to the manufacturer's instructions. The cDNA (complementary DNA) was stored at -80 ºC.

Primers

Primers were designed by Farquhar (2017) and are described below:
BYDV Forward 5' CGCAATGCCCAGCGCTTTCAG Reverse 5' CGCAATGCCCAGCGCTTTCAG RGMV Forward 5' GCTTCATGGTTTGGTGCATGG Reverse 5' GTGCCATTATTGACCGCAACG. These primers had a Tm (melting temperature) of 60 and 58°C, respectively, and yielded amplicons of 124 base pairs (bp) and 144 bp, respectively.

qRT-PCR (Illumina Eco™ Real-Time PCR System, Illumina, Inc., San Diego, CA, USA). This was used in conjunction with a Eppendorf epMotion 5070 liquid handling robot (Eppendorf Co., Hamburg, DEU, Germany) to quantify target transcripts within a given sample through interpolation from the standard curve, generated by creating a 10-fold dilution series across multiple $\log_{10}$ concentrations.

Establishment of a standard curve

Before qRT-PCR analysis, a PCR product of known concentration (1ng/µl) was used to create the dilution series and generate an 8-point standard curve for each assay, covering 7 orders of magnitude (1×10^{-1} - 1×10^{-8} ng/ul). Each dilution of known concentration was used as a standard, and tested via qRT-PCR as follows: 4 µl from each standard and 6 µl of master mix containing 0.2 µl of each primer, 0.6 µl PCR grade water, and 5 µl 2x TakaRa SYBR® Premix ExTaq ™ II PCR reagents, were added to each of the 10µl wells on a 48-well plate. Analysis of the dilution series using each primer pair was performed in triplicate. Thermo-cycling parameters were 95°C for 1 minute x 1 cycle, then, 35 cycles; denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 15 seconds, followed by melt curve analysis with denaturation at 95°C for 15 seconds x 1 cycle, annealing at 55°C for 15 seconds x 1 cycle and denaturation 95°C for 15 secs. All assays included a final incubation stage of 30°C for 30 seconds. The Illumina EcoStudy 4.0 software (Illumina, Inc., San Diego, CA, USA) was used to generate a standard curve, in which the mean detection threshold, quantification cycle (Cq) was plotted against the log quantity of the standard (Boonham *et al.* 2014). On the basis of the standard curve, the performance and efficiency of each PCR reaction was calculated e.g. for BYDV an 8-point standard curve was developed, which was detectable to 0.00001 ng template, and amplification measured within the in the range of 7-24 cycles (Cq). Outside of this range BYDV may be present but is not reliably detected.

qRT-PCR assays

These assays were performed on cDNA from the ryegrass samples by thermo-cycling (as described above) in triplicate. During each assay, triplicates of positive standards, plate calibrators (PC), and no-template controls (NTCs) were included to test for amplification, variability across plates, genomic contamination and PCR artefacts, such as primer-dimers.

Converting Cq values to copy number

Raw data obtained from qRT-PCR assays provided Cq values. These were converted into viral copy number (ng/ul of template) by back calculation from the slope of the standard curves as described by http://www.scienceprimer.com/copy-number-calculator-for-realtime-pcr.

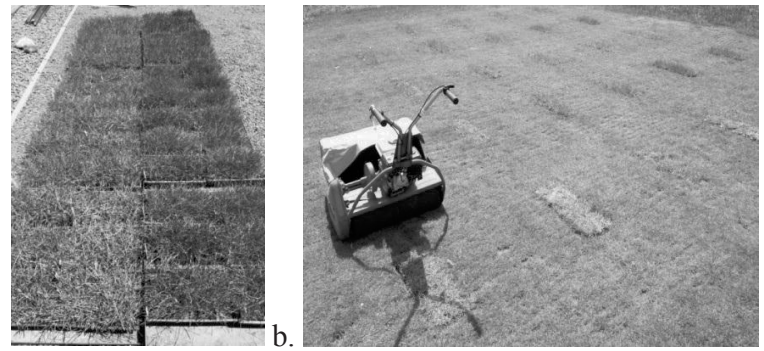

Figure 1 (a) Photograph of old (left) and young (right) transplants awaiting transfer to the trial block. (b) Photograph of the trial block depicting the sample swards in a turf grass background. Swards in foreground have been cut.

Statistical analysis

For the yield difference between old and young swards, one-way ANOVA of the combined yields of old versus young mini-swards was performed, and for yield assessment of old versus young between lines, a two-way ANOVA undertaken. For viral load, normalised copy number values obtained from each virus assay were subjected to a $\log_{10}$ transformation before repeated measurement ANOVA via GenStat version 18 (VSN International, Ltd). REML ANOVA analysis was adjusted for correlations. For BYDV, samples below the Cq threshold were adjusted by adding ½ the minimum value of the lowest copy number detected (data not shown). Significance was indicated by $P<0.05$ or less. Graphs reflecting the results of the REML ANOVA were generated using GenStat 18 software (VSN International, Ltd.).

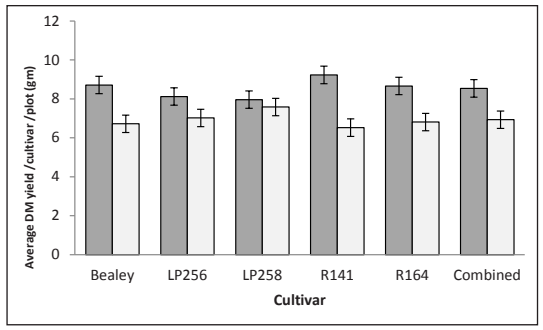

Figure 2 The average combined harvest sward DM yield of each line for young (dark bars) versus old (light bars) ryegrass ($P<0.05$, df 4; SED = 0.448; LSD = 0.946)

Results

Yield

Overall, the total yield (combined sward average DM of all old or new mini-swards/line number) of young ryegrass exceeded old ryegrass by 18.7% (42.7 g versus 34.7 g, $P<0.01$, $LSD_{5\%} = 0.46$). ANOVA showed that all lines except Lp258 had significantly greater ($P<0.05$) combined total yield for young ryegrass compared to old, and this was most extreme for line R141 with a 29% difference and least for LP258 with only a 4% difference (Figure 2). Analysis of the surrounding turf DM data indicated a slight effect on yield across the block but not enough to effect the trial outcomes (data not shown).

Persistence

The yield data in Figure 3 demonstrates a declining trend over time in both young and old treatments for all cultivars. The old treatment never exceeded the young for any cultivar at any time (except Lp258 on 9/12/2015) confirming a decline in yield over time. These data, combined with the observation that upon establishment the old swards looked paler compared to the young (Figure 1a), and visually at the end of the experiment, old swards had more ryegrass loss and greater weed ingress (not quantified), suggests that the old ryegrass, as well as yielding less, were less vigorous and persistent.

Viral load

The analysis of viral load showed that, overall, there was significantly more virus present in old swards compared to young swards ($P<0.001$) (Figures 4, 5). For RGMV the viral load in the young grass increased significantly between the April 15 and February 16 assessments, reaching near old infection levels by the end of the experiment. The exception was Lp258 which had levels similar at the start of the analysis ($P<0.006$ for viral load and sampling date and age, whilst sampling date and viral load interaction was $P<0.003$). The BYDV levels in the young swards showed little change, but in old swards BYDV levels dropped towards the end of the experiment, as old and young levels began to converge ($P<0.021$ for the interaction between viral load, age and sampling date). BYDV analysis gave readings below the quantifiable Cq threshold for 29/45 samples (3 reps for 3 times x 5 lines) for young, and only 8/45 for old

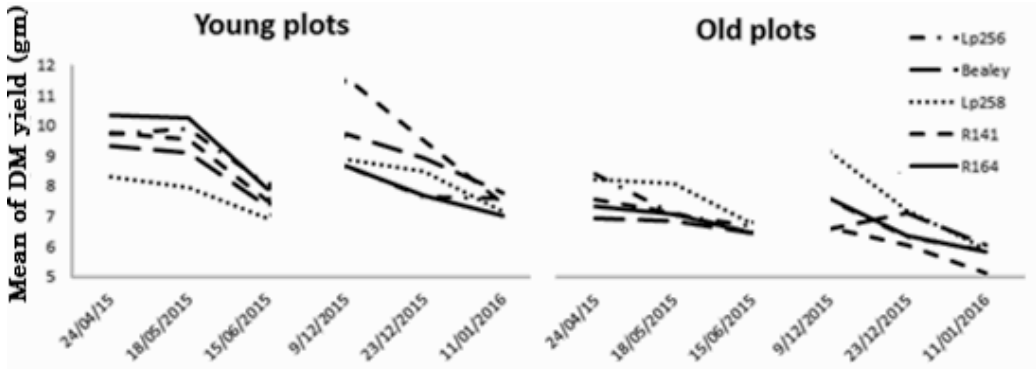

Figure 3 Yield over harvests for young (left) and old (right) ryegrasses

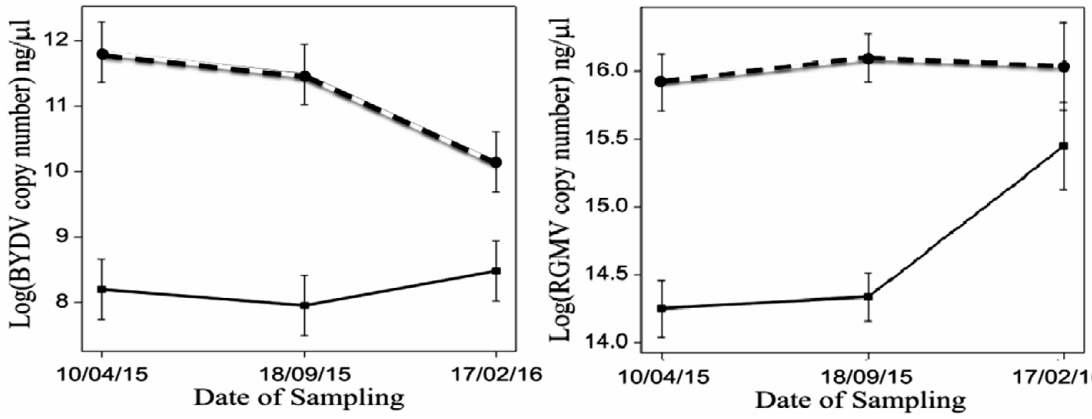

Figure 4 Average, BYDV (left) and RGMV (right) load, of all lines combined for samples above quantifiable Cq threshold, in old (dotted lines) versus young (solid lines) ryegrass for three samplings. Error bars are the standard error of the means.

(data not shown) again confirming higher levels in old. For old swards BYDV averaged 5 x 10^5 ng/ul template, whilst young averaged 2.1 x 10^5 (calculated by the sum of the counts/number of swards with Cq above threshold). For RGMV the levels of old averaged 1.2 x 10^7 whilst that of young were 4.9 x 10^6 and none of the samples were below the Cq threshold. Analysis of the old diploid RGMV lines (data not shown) revealed a trend between RGMV load and yield existed with R141 and R164 having the lowest yield and highest RGMV whilst Lp256 and 258 had lower RGMV and higher yields (R^2=0.34). This was not the case for BYDV.

Discussion

Age of sward

The serendipitous opportunity for this experiment arose from the existence of a 10 year old perennial ryegrass persistency trial and availability of the original seed. This created some caveats to the experiment and although tillers were washed before transplanting removal of existing pests and diseases on the old tillers could not be confirmed, so latent disease carry-over may have had some impact. Although by default only surviving genetic material was used in the old swards, a large yield loss was still observed in old versus young swards. This was consistent within lines, except in Lp258 where the difference was not significant. Interestingly, Lp258 also had comparable RGMV values between young and old swards from the first sampling. Other lines exhibited a significant 14-23% yield difference, whilst line, R141, was an outlier with a larger (29%) difference in yield. This line difference suggests a host genetic effect, though more research would be required to confirm this.

Persistence

The short (10 month) duration of the trial prevented within treatment persistency (yield over time) from being scrutinised as seasonal yield variation effectively masked the data. However, with the caveat that the genetic profile of old and new swards was not analysed, the old swards performed worse than the young. The observed decrease in vigour and yield of old swards was unexpected as by default plants were chosen because they had persisted for 10 years. This suggests that the original plants were somehow conditoned/

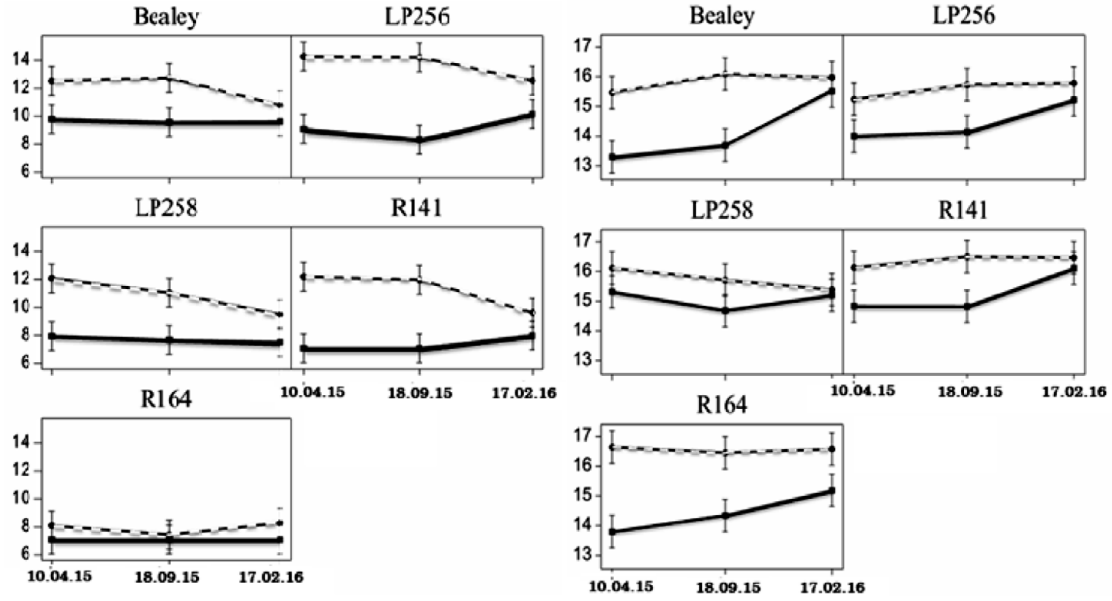

Figure 5 BYDV (left) and RGMV (right) load of samples above quantifiable Cq threshold in each line over three sampling times. Young swards (solid lines), old swards (dotted lines). Data are expressed as log of the copy number in ng/ul of the sample templates. Error bars are the standard error of the means of the 3 biological replicate swards for each line.

adapted to the enviroment they had been growing in and that this was not advantageous when transplanted into a new envronment or for biomass production. The role of epigenetics in such adaptation to environmental stresses is well documented (e.g. Baulcombe & Dean 2014); perhaps such epigenetic adaptations were compromised in the new environment.

It is important to highlight the difference between persistency and survival in this work. Here persistence has a commercial imperative that also requires a performance component (i.e. yield over time), whilst survival is just the ability to endure. It makes little difference to the grass if it adapts and commits reserves or sacrifices growth potential to combat virus, disease, and the environment, instead of providing them for the grazing animal in the form of leaf growth, so long as it survives to reproduce, i.e the grasses survival mechanism does not consider the farmer's requirement for leaf growth.

Viral load

Historically viruses have been detected by ELISA analysis (Webster *et al.* 1996) and whilst this method is easy to use, it is difficult to quantify virus load with previous researchers tending to publish the percentage of plants infected rather than sward viral load. The qRT-PCR method was chosen to provide better quantitative data, but ultimately proved technically challenging (Farquhar 2017). However, all the RGMV tests produced data above the Cq threshold enabling determination of load. The lower detection in BYDV (18% in young and 64% in old) possibly reflected lower infection levels or lower latent load. Trends were observed and significant differences in viral load, consistent with the hypothesis that increasing viral load could reduce yield, were demonstrated.

Old swards of diploid lines showed the trend for increasing RGMV load to be associated with decreasing yield, this did not hold true for 'Bealey', but as a tetraploid line it was expected to have a different viral load per unit of cell volume compared to diploids. The trend did not hold true with young material, but at initial lower infection levels, the host genetics may have had a greater impact on yield. Severe RGMV impacts on yield (up to 50% DM yield loss), quality and persistence have been reported (Wilkins & Hide 1976; Eagling *et al.* 1992; Guy 1993; Webster *et al.* 1999, 2005). The severity of impact varies due to viral strain, host genotype, incidence and the environment (Webster *et al.* 1999, 1996). In this study all swards were infected, which may be due to the location of the trial (a ryegrass breeding station), but it may also imply that the qRT-PCR method employed here is more sensitive than the previous methods used. As individual plants were not measured it was impossible to determine the percentage of infection, or level, within individual plants, although it is the response of the sward that the industry is ultimately concerned with.

For BYDV no relationship was observed between viral load and yield for any line in young or old swards, indicating, as suggested by Latch (1980), that BYDV is of less consequence to yield than RGMV. It was also

noted that BYDV within a sward often varied above and below the Cq threshold over time, suggesting a below threshold latent infection is present within swards, that may increase under particular conditions. This would fit with reports on the variable nature of BYDV incidence in New Zealand (Guy 2014). Historically, the impact of BYDV on ryegrass is reported as variable (Latch 1980; Clarke & Eagling 1994; Bisnieks *et al.* 2002), but BYDV yield losses of 22.4 to 24% have been reported (Wilkins & Catherall 1977; Latch 1980). Neither of these reports commented on the RGMV status of the ryegrass and so its impact could not be ruled out.

This trial showed an average yield deficit (18.7%) for pastures with raised RGMV and BYDV virus levels. Whilst the effect of age, epigenetic or other latent infections by virus, pests or diseases could not be ruled out, the data supports reports spanning 5 decades that highlight the potential damaging impact of these viruses on ryegrass yield. Despite this, it remains an under-studied area as little research seems to be aimed towards finding a solution to this issue.

Conclusions

The results indicate that; 1) old ryegrass plants yielded less than young; 2) at least two viruses exist in perennial ryegrass and; 3) viral load was greater in older material. The nature of the correlation between viral load and yield could not be established and age alone could not be ruled out as the cause of yield decline. Neither could potential epigenetic or genetic drift factors between the old and young swards or other diseases present in the old material. This and other research indicates that viruses and age (Robbins *et al*. 1987; Kibblewhite *et al*. 2014) have a negative impact upon plant yield, and that the interactions are dynamic, complex, and likely influenced by Genotype x Virus x Environment interactions over time. This study only looked at two viruses, yet many others can infect ryegrass (Guy 2014) and synergistic interactions can enhance impacts (Carfune *et al*. 2006). To eliminate confounding factors, including age, more controlled studies are required. However, this study, along with others over the past 5 decades does show that age and/or virus effects are likely related to DM yield decline in pasture. This should be of concern to the pasture industry as it may undermine current breeding efforts to improve long-term DM yield, if not taken into consideration.

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