final report

Project code: B.AHE.0040

Prepared by: Kevin FM Reed and Wade J Mace

Date published: February 2013

ISBN: 9781741919806

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Endophyte metabolites associated with severe cases of perennial ryegrass toxicosis

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

Concentration of endophyte metabolites were analysed to investigate the unique, severe expression of perennial ryegrass toxicosis (PRGT) in SE Australia. PRG was monitored at 4 Vic. sites, at Lincoln NZ and on VIC/TAS farms with stock experiencing perennial ryegrass toxicosis (PRGT). The 2010-11 summer in Vic. was atypically moist. Lolitrem B was consistently high at Lincoln and 2-3 times that observed in Vic. samples of iso-genetic PRG, or in PRG causing PRGT. It was a most significant toxin in 2011; in 2012 toxicosis was primarily ergovaline-based. Lolitrem B precursors were less concentrated in Vic. than in NZ PRG and in 2011-12 ergovaline concentrations were greater in Vic. Some unidentified metabolites were noted – in both regions. High solar radiation and its interaction with ingestion of vaso-constrictive ergot alkaloids, is considered important re PRGT in Australia.

Executive summary

Over two seasons, perennial ryegrass (cultivar Samson, naturally infected with the wild-type endophyte, *Neotyphodium lolii*) was sampled on 5 occasions during November to May at 4 farms in Victoria and at Lincoln, New Zealand. Endophyte frequency in the populations was 77-100%. Additionally, pasture on 20 farms with stock experiencing perennial ryegrass toxicosis (PRGT), was sampled; these pastures had endophyte infection frequencies of 95-100%. The 2010-11 season in Victoria was most atypical; pasture stayed green through summer. The following season was more typical. Analysis consistently showed high concentrations of lolitrem B at Lincoln which were significantly greater, by 2-3 fold, than those observed in Victorian samples of iso-genetic material and of the material associated with PRGT. PRGT samples commonly had concentrations of lolitrem B that were above tolerance levels in 2011 but in 2012, ergovaline was the dominant toxin; its concentration commonly exceeded tolerance levels.

To explain the severe PRGT that occasionally occurs in Victoria and Tasmania (viz. heavy losses of livestock including many due to sheep and cattle crowding into dams etc.), we investigated the possible importance of other endophyte metabolites - that appeared on HPLC chromatograms of Australian PRG samples from 2002 but not observed for toxic PRG samples taken in NZ - by collecting quality samples (the 2002 samples had been stored at ambient temperature for 6 years) and thereby ruling out compounds produced by advantageous fungi/mould/bacteria growing on stored samples. The presence of these other unidentified peaks was confirmed on HPLC chromatograms but their expression was not significantly different between NZ and Australian grown PRG and did not correlate with any of the known indole diterpene compounds/precursors detected by LC-MS/MS analysis. No unidentified peaks were observed in the erogvaline HPLC chromatograms for any samples on this occasion.

Mass spectrometry (LC-MS/MS) was carried out to determine indole diterpenes present in the lolitrem B biosynthesis pathway and for the ergot alkaloids present in the ergovaline pathway. The values for lolitrem B determined by LC-MS/MS correlated well with those obtained using HPLC. Significantly higher expression was observed in the New Zealand relative to Victorian samples of PRG for the indole diterpenes involved in the biosynthesis of lolitrem B, viz. paspaline, terpendole C, lolitrem E, lolitrem B and lolitrem F. For the ergot alkaloids, significant differences were not apparent between Vic. and NZ samples in year 1. In year 2, LC-MS/MS results showed ergovaline concentrations were greater in Vic. samples. The presence of ergotamine, ergocryptine and ergocornine (*Claviceps-purpurea* produced ergot alkaloids) was detected at 6/27. These ergot alkaloids should not be overlooked as contributing to toxicosis in stock grazing PRG.

Table of Contents

1. Background

Perennial ryegrass toxicosis (PRGT) causes considerable losses in production for cattle and sheep in Australia (Sackett & Francis 2006). The expression of acute PRGT in SE Australia includes high mortalities and dam crowding (Reed *et al*. 2005). Such extreme effects are not recorded in New Zealand and other countries. In NZ the levels of the known toxin, lolitrem B, reported in samples of toxic PRG are typically higher - and ergovaline levels often lower - than in Australia. Alkaloid analysis of stored toxic PRG tissue from Victoria revealed unidentified peaks that had not been observed in toxic PRG from NZ.

Most of the Australian districts where perennial ryegrass (PRG) is used for meat production have a climate quite different to the longer growing seasons and cooler temperatures common in New Zealand. Consequently, endophyte-produced toxin profiles may differ. No studies have yet been made into the range of toxins occurring in Australian PRG and their possible role in the distinct expression of perennial ryegrass toxicosis (PRGT) recorded there (Reed *et al.* 2005). New Zealand research has shown that lolitrem B and ergovaline are the most important toxins associated with PRGT but that *Neotyphodium* endophytes produce a wide range of metabolites, many of which are known toxins and tremorgens (Lane 1999; Lane *et al.* 1999; Panaccione 2005: Saikia *et al.* 2008). A more complete view of endophyte chemical ecology is required in examining why the extreme clinical PRGT signs and catastrophic, dam-crowding and losses that occasionally occur in southern Australia are not observed in New Zealand. Levels of the common toxins, ergovaline and lolitrem B, detected in toxic PRG in Australia are not substantially different to those found in toxic PRG in NZ.

In line with recommendations made by the PRGT Steering Committee in 2008, liaison that developed at MLA's 2009 PRGT R&D priorities workshop at MU, Werribee, led AgResearch chemists to examine, by HPLC, 16 PRG historic samples. These samples had been collected from several severe PRGT-affected farms in Victoria, 2002, which between them lost >6,000 head. The chromatograms for both ergovaline and lolitrem B revealed the presence of metabolites not normally observed in NZ toxic PRG (an example of which is shown in Figure 1). This result suggested there may be a unique profile difference between Australian and New Zealand toxic PRG.

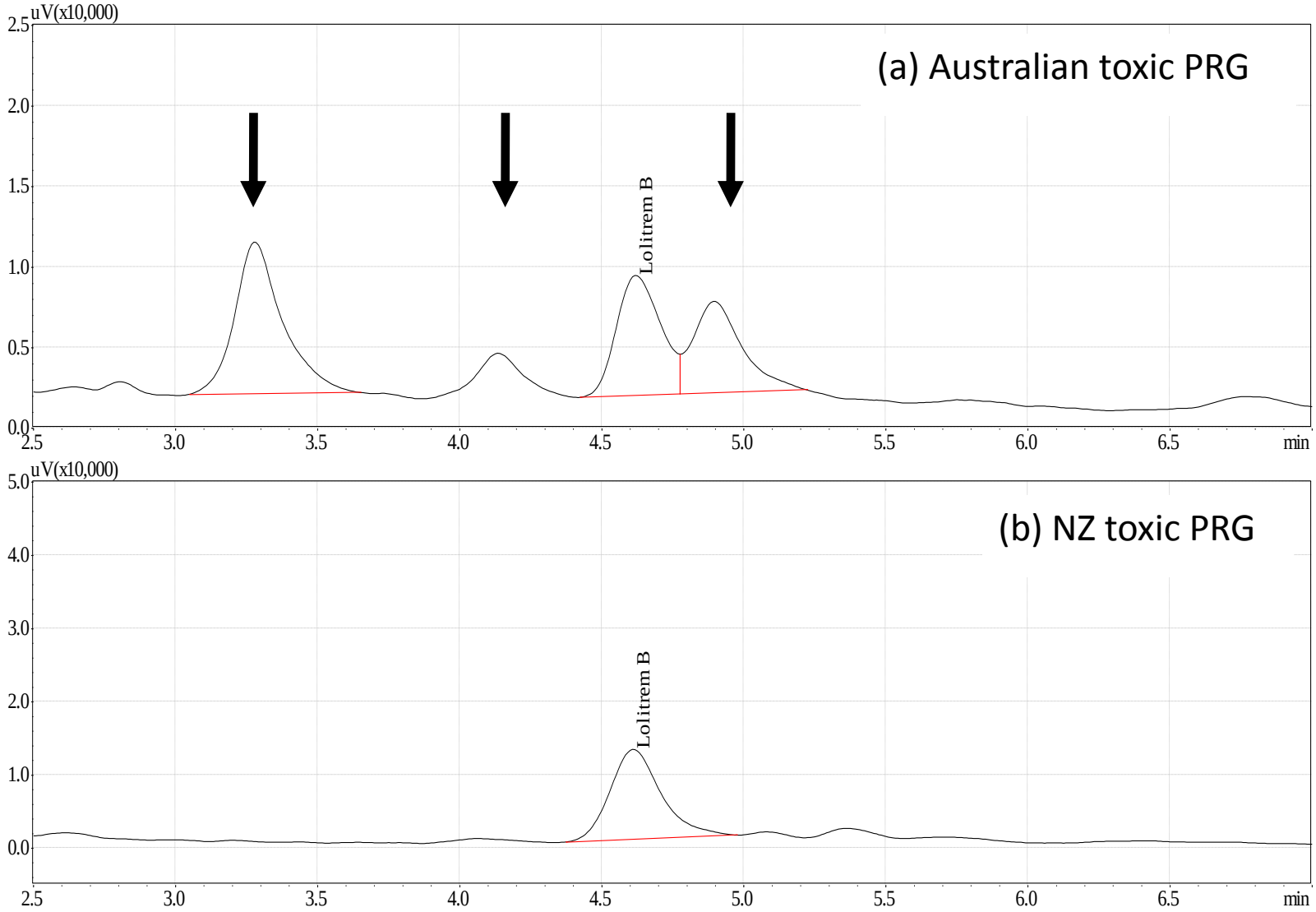

Figure 1: Lolitrem B chromatograms of representative (a) Australian and (b) New Zealand PRG samples. Peaks indicated by an arrow are not normally observed in New Zealand material.

2. Project objectives

1. Investigate variation in toxin synthesis in contrasting environments that may explain extreme PRGT phenomena in SE Australia. Monitor toxin pathway metabolites over two seasons in 3 replicates of a common cultivar of PRG (PRG, cv Samson, infected with wild endophyte) over the PRGT period at 3 Australian and 1 New Zealand site to compare baseline levels and variation in specific metabolites and their maxima that may help explain extreme toxicity. Analyse for ergovaline and lolitrem B (and for the full range of indole diterpenes and ergot alkaloids on one rep).
2. Identify any alkaloids in PRG that distinguish acute PRGT events in Australia from those recorded in NZ. Collect and analyse (as for (1) above) grass samples associated with extreme PRGT events. Compare alkaloid levels to those observed at the sites above (1). Additionally, take samples and analyse from similar nearby sites where livestock are not exhibiting PRGT (yet are grazing wild type endophyte-infected toxic PRG pasture). Examine samples for the presence of additional indole diterpene and ergot alkaloid metabolites that may distinguish herbage associated with severe outbreaks of PRGT and help explain the dramatic expressions of PRGT recorded in Australia relative to that observed in New Zealand.

NB. An optional further analysis (Study 3) was planned as a contingency - to investigate additional metabolites that may distinguish extreme PRGT – if evidence that variation in the expression of PRGT could not be explained by the results obtained in the first two studies.

3. Methodology

When sampling perennial ryegrass for endophyte frequency, we used a stratified pasture sampling technique, the entire plot or nominated paddock section was traversed in a zig zag (plot) or 2 diagonals basis (paddock) to collect tillers of perennial ryegrass for determining endophyte frequency. At least 40 perennial ryegrass plants were sampled at each site to determine endophyte frequency. Three tillers per plant were cut at ground level with the aid of a scalpel. Tillers were chilled immediately after cutting by putting them into an insulated chest containing frozen ice bricks (temperature 0-2°C) and subsequently stored at -18 to -20°C until tested for *Neotyphodium* endophyte infection frequency using a solid phase, stacked immunoblot assay (Hiatt *et al.* 1999) – this test was conducted by Paratech Veterinary Services, Wickcliffe, Vic. for Australian sites, or by AgResearch, Palmerston North, for the New Zealand site.

When sampling perennial ryegrass for determination of alkaloids, we used a stratified pasture sampling technique, the entire plot or nominated paddock section was traversed in a zig zag (plot) or 2 diagonals basis (paddock) to collect perennial ryegrass herbage for determining alkaloid concentrations. 25 perennial ryegrass plants were sampled (cut at ground level by scalpel) from each plot or nominated paddock section. Samples (fresh weight ~80-100g) included dead and green material and were chilled after cutting by putting them into an insulated chest containing frozen ice bricks (temperature 0 - 2°C). Samples were cleaned of any non PRG material, weighed and laid out and photographed. Samples were then freeze-dried and ground through a 1 mm screen and stored at -18°C to -20°C until analysed.

Alkaloid concentrations for the Australian sites were determined for 3 replicates (separate sample areas) by Southern Scientific Services, Hamilton, Vic., using high pressure liquid chromatography (HPLC) for lolitrem B (Gallagher *et al.* 1984) and ergovaline using a modification (Reed *et al.* 2004) of the methods of Shelby and Flieger (1997) and Shelby *et al.* (1997). Alkaloid concentrations for the New Zealand sites were determined by AgResearch, Palmerston North, NZ, using HPLC for lolitrem B (modification of the method of Spiering *et al* 2005) and ergovaline (modification of the method of Speiring *et al* 2002). HPLC analyses provided quantitative (and comparable between year) data and the opportunity to further evaluate the "unknown" and interesting peaks that were observed in 2002 samples (Reed *et al.* 2011b). Analysis of the indole diterpenes present in the lolitrem B biosynthetic pathway and ergot alkaloids was completed (one replicate) by AgResearch, Palmerston North, NZ on all Study 1 and 2 samples using mass spectrometry (LC-MS/MS).

Four Australian sites and one New Zealand site were monitored. The Australian sites were planned to cover a range of climates commonly associated with dominance by perennial ryegrass. We selected pure perennial ryegrass swards of a young age sown with a known cultivar (a similar cultivar across sites) and infected with wild endophyte. Sites with swards of cv Samson were selected at Camperdown, Dookie and Outtrim. The Camperdown site represents a lax grazed, stock-piled pasture while Outtrim at a similar latitude (with a short dry period) and Ballarat (80 km north of Camperdown) represented regular defoliation management. All the above mentioned sites except Dookie are variety trials where inputs have been tightly controlled, soil fertility maintained, genetic integrity is reliable and 3 replicate plots are available. The site at Dookie was an MLA-supported grazing experiment (1 ha plots and 3 replicates) on Melbourne University's farm, due to commence animal production measurements in February 2011 - with breeding ewes and weaned lambs.

For statistical analysis of Study 1 results, the method of restricted maximum likelihood (REML) was used with site and sampling date, where appropriate, fitted as fixed effects and year, site, replicate and harvest, where appropriate, fitted as random effects. All statistical analyses were performed using GenStat (GenStat Committee 2008). For the LC-MS data, comparisons between groups were made by one-way analysis of variance using MiniTab 16.

4. Results

Negotiations were completed with cooperators as to suitable perennial ryegrass sites and the sites were characterized for botanical composition, genetic integrity, endophyte infection and soil fertility - in N Victoria, SW Victoria, SE Victoria and New Zealand. Protocols were agreed with all cooperating workers. In year 1, considerable rain fell during early summer at the Victorian sites and growth of PRG continued through summer, a most atypical season for Victoria but similar to the usual NZ summer. Four Victorian and two NZ sites were sampled for alkaloid analysis in December, January, February, March and April and the samples described, freeze dried and ground. All 3-replicate samples were analysed as one batch at the end of the sampling period. The Vic samples were analysed at Southern Scientific Services, Hamilton, VIC and the Lincoln NZ samples were analysed by the Endophyte Chemistry Laboratory at AgResearch, Palmerston North, NZ. In a separate study not scheduled in the proposal, sub-samples from one replicate at each Vic site were sent to AgResearch as a between-laboratory comparison with Southern Scientific Services, Hamilton, Victoria. The analysis showed comparable values were generated at each lab.

Study 1 – Alkaloid monitoring

Details of the pasture and soil at the sites monitored for toxins are summarized in Table 1. Sampling in Years 1 and 2 was completed on 5 dates between Dec 2010 and May 2011 (Table 2) and Nov 2011 and May 2012 (Table 3). Sampling dates in Year 1 were slightly delayed in January due to paddock inundation and road closures after the January 9 storms which caused unprecedented flooding in Victoria. A description of the grass and its stage of development at each sampling was recorded and is summarized in Tables 4 and 5. Lolitrem B levels peaked at several sites at the last sampling in Year 1. So as to include the build-up and decline of toxin concentration, the sampling dates were varied in Year 2 in order to widen the period of monitoring; viz. commencing in November and continuing into May.

Table 1. Details of sampling sites and endophyte infection, Study 1

SITE	Dookie	Camperdown	Outtrim	Ballarat	Branxholme	Lincoln NZ
Manager	M Henry	S Kemp	S Kemp	J Sewell	K Reed	L Fletcher
Cv & 'plot' size (m)	Samson HE 50x200	Samson HE* 5x1.2	Samson HE* 5x1.2	Samson HE 4x1	Ecotype Vic. 35x50	Samson HE 35x50
Date sown	9.6.2010	April 2009	April '09	23.4.2008	1983	Spring '09
Endophyte freq. (%)†	83 (seed, 9.6.10) 87 (24.11.10)	96 (5.12.10)	97(13.12.10) 98 (28.3.12)	75 (2009) 77 (30.4.12)	100 (21.2.11) 100 (14.5.12)	85 (Nov. 09)
Soil test	15.12.10	19.1.11	13.12.10	20.7.11	27.9.11	24.1.2011
Soil texture	Brown clay loam	Brown loam	Red clay loam	Red clay	Brown clay loam	Templeton silt loam
Al saturation%	1.2	6.4	3.0	11.0	7.6	
Organic C %	1.5	6.8	5.3	4.2	3.8	
pH (1:5, $CaCl_{2)}$	4.8	4.7	4.6	4.5	4.6	
pH (1:5, water)	5.9	5.5	5.3	5.1	5.6	6.1
NO_3 N mg/kg	<1.0	4.0	27.0	5.9	4.2	
Soil P (Olsen) mg/kg	20	16	38	27	17	13 µg/ml
Soil available K mg/kg	240	120	290	300	131	237 µg/ml
Sulphate S (KCl 40) mg/kg	4.6	8.9	15.0	71.0	7.6	
K:Mg	0.3	0.3	0.4	0.9	0.3	
Electrical Cond'y. dS/m	0.05	0.08	0.11	0.17	0.06	

†date sampled shown in parenthesis *same seedline

Table 2. Pasture management, date of sampling, stage of grass development, grass height and proportion green at sampling, 2010-11

	Ballarat	Camperdown	Dookie	Outtrim	Lincoln, short	Lincoln, ungrazed
Pasture management events						
Mown	-	18 Feb	16 Dec	13 Dec, 12 Jan, 23 Jan	15 Oct, 13 Nov	-
Grazed	9 Dec 9 Feb	-	From 22 Feb	-	-	-
Predation	-	Slugs Dec-Apr	-	-	-	-
Date of sampling						
DEC	9	5	15	13	8 & 21	8 & 21
JAN	18	19	18	12§	19	19
FEB	8	12	13	11§	10	10
MAR	21	23	24	26	23	23
APR	19	25	28	23		
MAY					9	9
Stage* of grass development for primary/regrowth						
DEC	V§	R3	S3	R1		
JAN	V	S4‡	V§	R1		
FEB	V§	S4‡	V	V		
MAR	V	V§	V	S1‡		
APR	V	R1†	V	V		
MAY						
Ryegrass height (cm)						
DEC	40	90	90	22	9	70
JAN	18	65	20	35	12	70
FEB	20	70	17	18	12	70
MAR	35	9	10	48	14	70
APR	14	10	10	15	14	70
MAY					14	70
Proportion of grass green.						
DEC	1.0	0.9	0.8	1.0	0.9	0.8
JAN	0.9	0.15	0.15	1.0	0.8	0.7
FEB	0.9	0.1	0.5	0.9	0.8	0.5
MAR	1.0	0.2	0.1	1.0	0.8	0.4
APR	1.0	0.5	0.2	1.0	0.9	0.6
MAY					0.9	0.6

† aerial tillering from long stubble, ‡ lodged, V§ regrowth post-mowing,

* Codes describing stage of plant development (as per Moore *et al*. (1991)):
V vegetative, EO onset of stem elongation,

Floral development: R0 boot stage, R1 Infloresence emergence, R2 Spikelets fully emerged, R3 Peduncle fully elongated, R4 anthesis, R5 fertilisation.

Seed development: S1 – milk, S2 – soft dough, S3 – hard dough, S4 – endosperm hard.

Table 3. Pasture management, date of sampling, stage of grass development, grass height and proportion green at sampling, 2011-12

	Ballarat	Branxholme	Dookie	Outtrim	Lincoln
Pasture management events					
Mown				18 Oct, 9 Dec, 20 Mar	Not mown
Grazed		Continuously			Periodically
Predation			Snails, Feb		
Date of sampling					
NOV	10		18	13	30
DEC		15			13
JAN	9	19	10	4	18
FEB	22	21	10, 27	18	21
MAR	23	22		28	2, 29
APR	30		18		
MAY		15	4	6	
Stage* of grass development for primary/regrowth					
NOV	R1		R1	V	
DEC		S2			
JAN	R5	S4	M	S3	
FEB	M	M	M, M		
MAR	V	M		V	
APR	V		V		
MAY		V	V	V	
Ryegrass height (cm)					
NOV	80		15	32	11
DEC		25			6
JAN	52	25	8	16	5
FEB	6	20	6, 6	16	4
MAR	10	15		11	7, 4
APR	6		7		
MAY		8	8	30	
Green proportion					
NOV	0.95		0.65	0.95	0.8
DEC		0.02			0.5
JAN	0.9	0.01	0.05	0.8	0.3
FEB	0.1	0	0.03, 0.15	0.4	0.3
MAR	1.0	0.65		0.8	0.4, 0.4
APR	0.98		0.6		
MAY		0.05	0.8	0.99	

* coded as per Moore *et al*. (1991), see key under Table 2

Outtrim fertiliser applications, 2011-12: 18 Oct 250 kg/ha Pivot 400,
9 Dec 100 kg/ha Urea, 20 Mar 200 kg/ha Pivot 400.

In both years, ergovaline and lolitrem B concentrations in perennial ryegrass from Victoria were affected by site, and month of sampling. And for both toxins in both years, the site by month interaction was significant (*P*<0.001) (Table 4).

Table 4. Tests for fixed effects of site and month of sampling: F probability

Main effect	Table 5, 2010-11		Table 6, 2011-12	
	ergovaline	lolitrem B	ergovaline	lolitrem B
Site	<0.001	<0.001	0.037	<0.001
Month of sampling	<0.001	0.005	0.051	<0.001
Site x month	<0.001	<0.001	<0.001	<0.001

In year 1, the concentration of lolitrem B in PRG peaked in summer at all sites and it then rose again in autumn at all sites except for the short pasture at Lincoln. Apart from the Ballarat site, it exceeded the tolerance level (1.8 mg/kg) in summer. The lolitrem B concentration at Lincoln remained above the tolerance level throughout the sampling period and was markedly higher than that recorded for the Australian samples.

The ergovaline concentration in PRG also exhibited two peaks at Dookie, Outtrim and Lincoln (tall). At Lincoln (short) remained above the tolerance level (0.8 mg/kg) through late summer and autumn and at Camperdown it remained above tolerance until late autumn. Ergovaline concentration only reached the tolerance level in December at Dookie and in January, and again in April, at Outtrim. Maximum ergovaline concentrations were recorded in summer where tall pasture had accumulated, viz. Dookie, Camperdown and Lincoln (tall). At the other sites the highest ergovaline concentrations were observed in autumn. Ergovaline concentrations for the Lincoln (short pasture) were slightly lower than for Australian sites in summer but not in autumn. For the ungrazed material, Lincoln (tall), they were high and remained at or above the tolerance level (0.8 mg/kg) throughout the sampling period (Table 5).

The results emphasise the significance of ergovaline in tall pasture - where seedhead and stem is present - and the relative importance of lolitrem B in short pasture.

Table 5. Concentration of ergovaline and lolitrem B in perennial ryegrass (mg/kg DM) –mean of 3 replicates (1 rep re Lincoln), 2010-11

2010-11	Dec	Jan	Feb	Mar	Apr-May	Mean
lolitrem B						
Ballarat	1.29	1.30	1.47	1.20	1.72	1.40
Camperdown	1.08	2.18	1.56	1.61	1.51	1.58
Dookie*	3.07	2.13	1.12	2.04	2.39	2.15
Outtrim	1.71	1.88	2.80	2.44	3.42	2.45
Mean	1.79	1.87	1.73	1.82	2.26	1.89
lsd (*P*=0.05)	Site: 0.314 Month: 0.278 Site x Month: 0.564					
Lincoln - short	1.84	3.06	4.16	7.75	4.44	4.25
Lincoln - tall	3.08	2.88	3.00	3.11	3.48	3.11
ergovaline (inclu. ergovalinine)						
Ballarat	0.16	0.21	0.24	0.27	0.60	0.29
Camperdown	0.45	2.17	1.01	0.44	0.32	0.87
Dookie[1]	0.81	0.16	0.09	0.18	0.20	0.29
Outtrim	0.18	0.42	0.34	0.27	0.75	0.37
Mean	0.40	0.74	0.40	0.28	0.47	0.46
lsd (*P*=0.05)	Site: 0.139 Month: 0.132 Site x Month: 0.264					
Lincoln - short	0.30	0.37	0.49	0.77	0.56	0.50
Lincoln – tall	4.76	3.45	0.79	1.59	1.08	2.33

*Ewes affected with PRGT in March-April

In year 2 the regular monitoring of alkaloids in Samson PRG was carried out again at 3 sites in Victoria as well as at Lincoln in New Zealand over at least 5 dates during the November-May period. Results are summarized below (Table 6). At Branxholme, a 4th site in Victoria, an ecotype Victorian PRG pasture with a PRGT reputation was also monitored. This site was included in place of the Camperdown Samson site sampled in the previous season.

In what was a more typical Victorian season than the unprecedented “green summer” that we experienced in year 1, ergovaline exceeded the tolerance level of 0.4 mg/kg (cattle) on 11/20 occasions. It exceeded the sheep tolerance level (0.8) on 2/20 occasions. These results were similar to the previous season (8/20, 3/20) and do suggest that ergovaline toxicosis is likely to be a more common occurrence than lolitrem B toxicosis. The above-tolerance ergovaline concentrations occurred on the two grazed pastures (Dookie and Branxholme) during the period when seedhead was significant. On the regularly cut (closely utilized) pastures, these levels occurred throughout the season. As in the previous season, ergovaline levels were relatively low at the (hot) Dookie site. At Lincoln, ergovaline concentration did not show a high peak but slightly exceeded 0.4 on 3/6 occasions. Ergovaline concentration in PRG peaked in early autumn at Outtrim and in summer at Branxholme and Dookie. Concentrations varied only slightly around the tolerance level at the other sites during the period of sampling (Table 6).

Lolitrem B exceeded the tolerance level (1.8) on only 2/20 occasions; both these critical levels, which were at Outtrim and Ballarat, were observed in autumn, following heavy rain. Both these sites had been fertilised with nitrogenous fertiliser in the preceding December and June respectively (Table 3). The low frequency of high levels was quite in contrast to the previous (New Zealand-like, green summer) season where lolitrem B was above tolerance in 9/20 occasions. In PRG pasture at Lincoln lolitrem B again exceeded tolerance levels (max 3.7) throughout December-March.

In year 2, the concentration of lolitrem B in PRG, similarly to year 1, peaked in summer at all sites and again in autumn at all sites except at Lincoln where the two peaks occurred early and late in summer. Again the lolitrem B concentration at Lincoln remained above the tolerance level throughout the sampling period and was markedly higher than that recorded for the Australian samples. At the Australian sites, the concentration only reached the tolerance level at two sites, both in autumn – viz. Dookie and Outtrim.

Table 6. Concentration of ergovaline and lolitrem B in perennial ryegrass (mg/kg DM) mean of 3 replicates (1 rep Lincoln), 2011-12

2011-12	Nov-Dec	Jan	Feb	Mar	Apr-May	Mean
lolitrem B						
Ballarat	0.31	0.75	0.52	0.36	0.93	0.70
Branxholme	1.14	0.56	0.41	0.58	0.50	0.76
Dookie	1.27	0.74	0.60	1.83	1.79	1.18
Outtrim	0.98	1.72	1.27	2.16	1.36	1.62
Mean	0.93	0.94	0.70	0.99	1.15	1.00
lsd (*P*=0.05)	Site: 0.413 Month: 0.413 Site x Month: 0.413					
Lincoln	2.45	1.93	4.18	3.43	3.22	3.04
ergovaline (inclu. ergovalinine)						
Ballarat	0.29	0.55	0.47	0.30	0.54	0.42
Branxholme	1.09	0.60	0.34	0.35	0.33	0.53
Dookie*	0.73	0.33	0.16	0.37	0.29	0.36
Outtrim	0.42	0.46	0.70	0.95	0.65	0.62
Mean	0.63	0.49	0.42	0.46	0.47	0.41
lsd (*P*=0.05)	Site: 0.300 Month: 0.324 Site x Month: 0.324					
Lincoln	0.50	0.22	0.51	0.51	0.37	0.42

*Ewes affected with PRGT in March-April

Study 2 – Perennial ryegrass associated with cases of PRGT

The year 1 following well above average summer rainfall, toxin levels, recorded at the Victorian sites monitored in Study 1, were high compared with those reported in the previous 4 years; they exceeded sheep/cattle tolerance levels on several occasions. It was a season without precedent; PRG remained green and continued to grow throughout summer and autumn. Reports of 'staggers' were received in early January and acute PRGT was expected. Autumn proved relatively cool and moist however, and the problem from PRGT was not too severe. It was a concern in parts of south west Victoria during March 2011 and, at the Dookie site, PRGT signs persisted throughout both autumn and early winter.

We investigated a sample of cases (Table 7). In March, producers had serious concerns when they went to move stock. As the use of supplementary feed was insignificant they had not had the same opportunities to see stock extended and were surprised by the high numbers of sheep that staggered and collapsed when stressed by mustering. Reports were received from across southern Vic. and Tas. Charles Sturt University staff and Hamilton Veterinary consultant D. Randell helped with some joint investigations. Eleven Hamilton district properties were visited, mainly in March. Pasture was described and grass was sampled. Stock were inspected and effects noted in consultation with vets and property managers. The CSU team collected brain and other tissues for pathology and biochemical investigations - and in some cases they collected the grass samples. The toxicosis problem declined into April as fresh growth occurred following the cool, wet conditions.

Seven pastures that were associated with PRGT on six farms were investigated in Study 2 and the associated PRG was sampled and analysed for ergovaline and lolitrem B. The concentration of ergovaline and lolitrem B in the PRG were above tolerance levels in one and seven of the pastures respectively. Another five pastures from different farms were later investigated by the CSU team who provided grass samples which we included in our analyses. Toxin concentrations were below the tolerance level in all five cases. This result may reflect sampling method – e.g. Concentrations are sensitive to height of cutting and botanical composition – and/or to the gap between the dates of peak PRGT signs and sampling.

As with the regularly sampled study 1 sites, it was ergovaline rather than lolitrem B that exceeded cattle tolerance levels in 11 of the 12 pastures investigated in year 2 (Table 8). And again, as with the regularly sampled sites, this was in contrast to the previous season. Landowners described the PRG as Victorian ecotype material in 9 cases and as cv Ellett in 3 cases. The cv Ellett pastures were at Hunterston.

Table 7. Study 2, 2011 cases of PRGT investigated, SW Vic. showing mean ergovaline and lolitrem B values (mg/kg DM)

Sample date	Farm, Locality	Paddock ID	Mean ergovaline	Mean lolitrem B	Synopsis, livestock effects*
14 Jan	*UL* Hamilton	D	1.42	2.74	15% down at moving. 1 dead after dipping
21 Feb	*W* Branxholme	P	0.52†	1.89†	30% with signs. 18/750 dead. 85 nursed
8 Mar	*FD* Melville Forest	PH	0.57	2.31	10% with signs
8 Mar	*HV* Melville Forest	R	0.33	2.57	20% with signs. 6 stayed down after gentle shifting
8 Mar	*HV* Melville Forest	M	0.22	1.88	80% of cows & calves with temperament at preg testing; 20% with staggers. 87% pregnant. 1 euthanised (down 5 days)
9 Mar	*C* Branxholme	J	0.31	3.20	6% down at moving. 6 down after 5 d
9 Mar	*MC* Caramut	C‡	0.17	1.38	7% down at moving
15 Mar	*R* Woodhouse	CY	0.42	2.69	10% down at moving (50% if dog). Cattle with temperament – extreme problems loading trucks
30 Mar	*M* Hamilton	LFW‡	0.11	1.33	1450 ewes. 43 drowned (dam with firm banks). 28% with abnormal gait, 17% down at moving. 15 remained down and died/euthanised
30 Mar	*K* Coojar	WF‡	0.29	1.44	30% with signs, 5% down, 5 still down after 3 days, 1 died
30 Mar	*W* *B*ranxholme	M‡	0.22	0.75	10% *c.* abnormal gait. Severe PRGT 6 weeks previously (shearing)
31 Mar	*M* Caramut	CM‡	0.26	1.00	25% *c.* abnormal gait
Wild endophyte-infected pasture sampled as a 'control' reference					
8 Feb	*UL Hamilton*	4	0.67	1.86	ungrazed
7 Mar	*UL Hamilton*	4	0.83	2.43	2.4% sheep with RGS
5 Apr	*UL Hamilton*	4	0.19	1.84	No signs
14 May	*UL Hamilton*	4	0.14	1.03	No signs

* euthanasia performed and post mortem information collected by Dr D. Randell (Hamilton) and Drs. J. Quinn & M. Comb, Charles Sturt University

† (Lolitrem B = 4.38 for green fraction & 1.43 for dry fraction. Similarly, ergovaline = 0.99 and 0.20)

‡ grass samples not subjected to botanical analysis

Table 8. Ergovaline and lolitrem B concentrations in PRG associated with PRGT, 2011-12

Year	No. of PRGT Pastures sampled	Ergovaline >0.4 (cattle)	Ergovaline >0.8 (sheep)	Lolitrem B >1.8	Lolitrem B >1.8 and ergovaline >0.4
2010-11	7	4	1	7	4
2011-12	13	12	2	4	4
Total	20	16	3	11	8

Two trips were made to Gippsland where six pastures were investigated. These were at Woodside (3), Hunterston (2) and McLaughlan's Beach. Five pastures were investigated in SW Victoria at Hawkesdale (4) and Birregurra. One PRGT-causing pasture was investigated and sampled at Cressy, Tasmania (Table 9).

Table 9. Study 2 – PRGT cases, 2012, showing mean ergovaline and lolitrem B concentrations in PRG (SSS HPLC analyses; mg/kg DM)

Sample date	Farm, Locality	Paddock ID	Endo-phyte freq. (%)	Mean ergovaline	Mean lolitrem B	Synopsis, livestock effects*
17 Feb	*P* Woodside	DFn	100	0.46	1.09	800 Mo ewes & lmbs. 10% with staggers, 50 died inclu 14 drowned
17 Feb	*P* Woodside	AH	nd†	0.25	1.32	Mo hoggets staggers. Scours despite Triguard drench
17 Feb	C Hunterston	J	nd	0.60	1.71	XB ewes w 240 lmbs ready to mkt when down w staggers, 20 drowned.
17 Feb	C Hunterston	ACne	nd	0.52	2.80	XB ewes w 240 lmbs ready to mkt when down w staggers, 20 drowned.
17 Feb	*W* Woodside	C	95	0.43	1.70	A few deaths in 500 Mo wnrs but pasture only 40% PRG. Ergot problem - *Claviceps phalarides*
17 Feb	*W* McLaughlans Beach	B	nd	0.45	1.54	Slight staggers; lost 5/600 Merino ewes
7 Mar	*BP* Birregurra	10	100	0.58	1.19	Lost 1/140 Angus wnr steers, 3 staggers. Only 40% PRG in pasture. Ergot problem – *Claviceps phalarides*
8 Mar	*B* Hawkesdale	12	100	0.48	1.55	30% of XB wnrs down at moving (13% had to be trucked). Falling even when not moved
8 Mar	*B* Hawkesdale	18	100	0.53	1.21	5% of 3YO Angus cows hypersensitive
8 Mar	*C* Cressy TAS	F19	nd	0.58	0.90	Delayed turn-off for 600 XB lmbs. 5 dead (3 drowned) 50 very poor; 50% with low LWG
30 Mar	C Hunterston	AC ne	100	0.68	1.87	500 lost from Facial Ecz. since the Feb sampling (for 2nd time in 3 yr)
2 May	*B* Hawkesdale	SWH	100	1.00	2.11	10% of Mo wnrs down at muster
2 May	*B* Hawkesdale	S	100	0.89	1.91	10% of Mo wnrs down at muster

* Note that PRG sampling was often 3-6 weeks after PRGT signs were first observed by the meat producer. † Not determined

The lolitrem B values of all the Year 1 samples of PRG that were analysed by LC-MS/MS were strongly correlated with those obtained using the classical HPLC analysis (R^2=0.91).

The mean Year 1 indole diterpene values from Studies 1 and 2 for VIC and NZ are shown separately, together with the values for Study 2 – both PRGT cases and the control pasture – in Table 10. The higher mean concentration of ergot alkaloids for NZ compared with Vic samples is associated with a much higher proportion of tall pasture samples in the New Zealand set (viz. 50% cf. ~5% Vic. set).

Table 10. Mean concentrations of indole diterpenes in PRG 2010-11 samples as determined by LC-MS (TSQ) and expressed as normalized areas.

Indole diterpene	Study 1 VIC	Study 1 NZ	Study 2 PRGT	Study 2 Control	CSU PRGT
No. of samples	18	12	21	7	11
1,3-Desoxypaxilline	47	152	132	53	82
Paspaline	461	1001	861	591	618
Terpendole C	124	308	206	132	142
Lolitriol	271	529	625	404	478
Lolitrem F	265	491	297	170	114
Lolitrem B	6053	9953	6825	4799	3783
Lolitrem E	263	464	384	196	166
Lolitrem A	1001	1376	931	718	590
TOTAL	11137	18321	13961	9440	7705

Statistical analysis of the indole diterpene data for year 1 showed significantly higher expression in the NZ relative to Vic samples (which included Study 2 samples) - for paspaline, lolitrem E, lolitrem B, lolitrem F (a stereo-isomer of lolitrem B) (P<0.004) and terpendole C (P<0.025) (Table 11). Paspaline, terpendole C and lolitrem E are directly linked to the synthesis (and expression) of lolitrem B.

Table 11. Mean expression levels for indole diterpenes showing significant difference in expression between Australia and New Zealand samples. Values are expressed as normalized area.

Indole Diterpene	Paspaline	Terpendole C	Lolitrem F	Lolitrem B	Lolitrem E
Mean, Australia - Studies 1 & 2	664	160	240	5825	288
Mean, New Zealand	909	305	434	8835	417
P-Value	0.003	0.025	0.004	0.001	0.003

Analysis of the ergot alkaloids (ergovaline biosynthetic pathway): A similar analysis was undertaken for the ergot alkaloids present in the ergovaline biosynthetic pathway. Statistical analysis showed that all were slightly higher in the NZ samples (P<0.10); the greater level observed for lysergol alanine was highly significant (P<0.01) (Table 12).

Table 12. Mean ergot alkaloid and peramine concentrations for Vic (Studies 1 & 2) and NZ samples of PRG determined by LC-MS (TSQ). Concentrations are expressed as normalized area

(ND = Not Detected)

Alkaloid	Vic	NZ	*P*
Chanoclavine	0.41	0.66	0.06
Agroclavine	ND	ND	-
Elymoclavine	ND	ND	-
Lysergol	ND	ND	-
Lysergic acid	<0.2	<0.2	-
Ergovaline	0.69	2.17	0.08
Ergine	0.90	1.45	0.10
Lysergol alanine	1.01	2.04	0.01
Total	3.4	6.7	
Peramine	11.7	17.5	0.06

Statistical analysis of the endophyte alkaloids data for year 2 showed significantly higher expression in the NZ relative to Vic samples (Study 1 samples) - for peramine, lolitrem B and its precursors - terpendole C, lolitrem A and E (Table 13). Ergovaline was significantly lower in the NZ samples. For the study 2 samples, lolitriol, lolitrems A, B and E, peramine and ergine were all greater than the values observed in study 1.

Table 13. Mean concentration of endophyte alkaloids in PRG for 2011-12 sampling groups (normalized areas) with the P value from ANOVA.

Endophyte Alkaloids	Study 1 VIC	Study 1 NZ	Study 2	P-value
No. of samples	21	6	36	
Paspaline	0.4934^{B}*	$0.6655^{A,B}$*	0.8390^{A}*	0.029
Terpendole C	0.0607^{B}	0.2477^{A}	0.1684^{A}	0.026
Terpendole N	1.134^{B}	$1.267^{A,B}$	1.870^{A}	0.081
Lolitriol	1.405^{B}	1.580^{B}	3.773^{A}	0.000
Lolitrem B	9.172^{B}	20.503^{A}	15.794^{A}	0.000
Lolitrem E	0.1959^{B}	0.5597^{A}	0.5574^{A}	0.001
Lolitrem A	1.024^{C}	3.762^{A}	1.658^{B}	0.000
Peramine	1.777^{C}	3.988^{A}	2.607^{B}	0.002
Chanoclavine	0.10389^{A}	0.08902^{A}	0.15413^{A}	0.106
Ergovaline	0.3870^{B}	0.2671^{A}	0.5882^{B}	0.028
Ergine	0.4298^{B}	0.2495^{B}	0.8675^{A}	0.007
Lysergyl-alanine	0.3779^{A}	0.3866^{A}	0.4829^{A}	0.217

*Means that do not share the same superscript differ significantly
(5% confidence interval, Fisher's LSD procedure)

Non-endophyte mycotoxins

In year 1, although the proportion of dead perennial ryegrass was unusually low in the unusually good summer for growth of grass, mature material was present – especially where growth became rank. The humid conditions probably favoured saprophyte activity and the presence of *Claviceps purpurea*-produced ergot alkaloids was detected in some samples. Private samples of mixed grass pasture submitted from Vic. vets also tested positive for other ergot alkaloids this last autumn (L Walker, Southern Scientific Services, pers. comm.).

Claviceps purpurea mycotoxins contaminated PRG samples again in year 2. In year 1, ergotamine, ergocryptine, ergosine and sometimes ergocornine were detected in 2 samplings at one monitor site (Camperdown) and in 2 PRGT paddocks (Woodhouse and Hamilton) (i.e. at 3/11 sites), and, in year 2, at 1 monitor site (Outtrim) and in 2 PRGT paddocks – Hunterston and Woodside (i.e. 3/16 sites).

Ergot was also confirmed on two "PRGT"- investigated (rank) pastures that contained rank *Phalaris aquatica* contaminated with *Claviceps phalarides*. Preliminary surveys (Reed and Moore 2009) have indicated that the presence of *Fusarium*-produced and other sources of mycotoxins (e.g. dioxynivalenol, zearalenone) are not uncommon in high rainfall zone pasture especially; they often co-occur there with ryegrass endophyte toxins.

5. Discussion

AgResearch's centre at Lincoln New Zealand is the leading centre for studying biosafety of perennial ryegrass endophyte associations and for research into endophyte effects on lamb production. Our results show that for cv Samson PRG infected with wild endohyte, sampled at similar times and under similar management, the concentration of lolitrem B and its precursors was considerably greater in Lincoln NZ samples than that observed in samples from Victoria. The difference was consistent and considerable. Concentrations remained well above the tolerance level throughout the 5 month sampling periods in NZ – in both years of the study. The NZ lolitrem B concentration was greater too, than that observed in samples of ecotype Victorian and cv Ellett PRG, similarly infected with wild endophyte, collected from Victorian farms reporting PRGT problems over the same period. This simultaneous monitoring of iso-genetic PRG confirms published reports of independent studies that were previously conducted in both countries and reviewed (Reed *et al* 2011b). That literature generally showed that the concentration of ergovaline was higher in Australian PRG than that reported in NZ research and our results provide some evidence to support that difference, viz. LC-MS data, year 2, but the difference was not as dramatic as for lolitrem B. This difference was quite reversed in year 1 where mature PRG was left ungrazed at Lincoln.

To address the objective of explaining the severe PRGT we observe in Victoria and Tasmania occasionally, when heavy losses of livestock occur including many due to sheep and cattle crowding into dams, we were curious as to the possible importance of other endophyte metabolites that appeared only from Australian PRG samples on HPLC chromatograms – not on toxic PRG samples taken in NZ. The original Vic samples taken from severely affected pastures in 2002 were not analysed by LC-MS/MS; no information can be gathered as to whether these peaks were ergot or indole diterpene related compounds. The reason for undertaking this present research was to obtain good quality samples (the 2002 samples had been stored at ambient temperature for many years) to rule out compounds produced by advantageous fungi/mould/bacteria growing on the

stored samples. Additionally this study provided side-by-side comparison of the same cultivar grown in both environments (Victoria vs. New Zealand).

The LC-MS/MS data we have presented here for the current two years samples (2010-11 and 2011-12) is also "targeted" analytical data, designed to pick up all of the ergot and indole diterpene compounds that we have seen previously. The LC-MS/MS analysis was not designed to be an exercise in searching for new compounds - that would have been the task of the proposed optional Study 3 analyses. The purpose of the LC-MS/MS analyses was simply to determine if the expression between NZ and Australia was significantly different for any of the alkaloids (which it showed there was).

The purpose of doing the lolitrem B and ergovaline HPLC analyses was to get quantitative (and comparable between year) data for these compounds, but also to further evaluate the "unknown" and interesting peaks that were observed in the 2002 samples. Was their expression significantly different between NZ and Australia and did they correlate with any of the known compounds (detected in the LC-MS/MS analyses)? The results showed no correlation between these compounds and the known indole diterpenes (no peaks were observed in the erogvaline HPLC analyses this time). There was no distinction between the NZ and Australian PRG samples regarding expression of these peaks.

These unusual peaks were similarly expressed in NZ and Australian PRG> We saw no evidence to suggest that they were associated with PRGT. It would take significant work to identify these compounds, even to the extent of determining whether or not they are indole diterpenes. For the above reasons, we decided their further investigation (i.e. identifying them by LC-MS/MS, and quantifying them more accurately) was not merited.

Our results suggest that the ergovaline: lolitrem B ratio, which can be expected to be greater in Victoria than in NZ, is likely to be more important than other metabolites in explaining the different expression of PRGT between the regions. The relatively high solar radiation and temperature expected in Victoria compared with NZ will result in more severe heat stress on ruminants and so aggravate the well documented effects of the vaso-constricting ergot alkaloids on animal physiology.

6. Conclusions

The ergovaline: lolitrem B ratio, which from our various Victorian surveys can be expected to be greater than that reported in overseas studies, notably NZ and Oregon USA, seems likely to be more important than other metabolites in explaining the different expression of PRGT between the regions. The relatively high solar radiation and temperature expected in Victoria compared with NZ will result in more severe heat stress on ruminants and so aggravate the well documented effects of the vaso-constricting ergot alkaloids on animal physiology. We noted that staggering *per se* was observed in some flocks/herds where lolitrem B < 1.8 mg/kg. This may reflect a lowering of the neurotoxin by the date of sampling – i.e. sampling post-date of peak occurrence. It may also imply a synergistic effect where the animal's tolerance of lolitrem B may be lowered with exposure to elevated ergovaline.

Over the two year investigation, toxic, non-endophyte ergot alkaloids were detected at ~22% of all the sites that we sampled. These then cannot be overlooked as a significant contribution to toxicosis from PRG pasture.

Australia's high solar radiation and temperature, and the interaction of this with the ingestion of ergot alkaloids, must be considered most relevant to the unique and severe expression of PRGT in Australia. And, without ignoring its unknown but possible synergistic effect with ergot alkaloids, the concentration of neurotoxic indole diterpenes *per se* in Australian PRG appears to be of less relevance.

7. Recommendations

1. Research is warranted to study the effects of ergot alkaloids in perennial ryegrass on animal physiology as the economic loss associated with their ingestion has been shown to be an extremely serious problem - in both endophyte-infected tall fescue and in *Claviceps*-infected feed. Their sub-clinical impact on livestock production and reproduction is likely to be significantly greater in Australia than in New Zealand where most of the biosecurity R&D on ryegrass endophyte has been conducted over the past 30 years. If confirmed, the animal health and economic implications need to be widely publicized and extended.

2. Selected "safe" endophytes are now inserted in new cultivars of PRG, some of which produce ergovaline but not lolitrem B - because ergovaline is an important insect pest deterrent. The development of experimental varieties of endophyte-infected PRG by AgR now make it feasible to cost-efficiently provide, as seed, separate sources of ergovaline and lolitrem B. Thus it will be possible to spike feed appropriately in order to study the separate effects of each toxin – and importantly, their synergy. Such feeding studies would unravel the relative impact of the two main toxins and help develop targets for PRG improvement and such therapeutic strategies that should help producers manage PRGT.

3. We suggest research into therapy that can prevent and lower the impact of PRGT is likely to be worthwhile as the vast areas of persistent, high-endophyte infected, naturalized PRG pasture in southern Vic. and Tas. are likely to be difficult to replace within the current economic framework.

8. Technology Transfer

The project was outlined in MLA *Feedback* in 2010 and in the *Western District Farmer* (2011) and *Hamilton Spectator* (2011). Kevin Reed attended the Grassland Society of Southern Australia's annual conference in 2011 where he spoke with several producers and advisors about the project. Early in 2011 we registered with the Best Wool Best Lamb and Best Beef Victorian networks advising them of our current project and our desire to meet with graziers who have experienced severe outbreaks and/or who wish to plan for containing the risk. Also in 2011 Kevin Reed visited the Veterinary and Agriculture Schools at Charles Sturt University, Wagga Wagga and discussed endophyte topics with staff there - and at I&I Wagga Wagga. During the project, three of the associates published scientific papers relevant to the research topic (see Bibliography).

9. Acknowledgements

The most unusually strong growth of perennial ryegrass throughout spring and summer in 2010-11, followed by the moderately severe onset of PRGT and the interest by CSU resulted in a greater than expected number of significant grass samples (most with linked clinical information from CSU). We consulted MLA re increasing numbers for analysis and appreciate their cooperation with that request. We thank Lester Fletcher, AgResearch, Stuart Kemp, PastureWise, Meredith, Vic., Reg Hill, James Sewell and Derek Mason, PGG Wrightson Seeds Australia, Ballarat and Michelle Henry, University of Melbourne, Dookie Campus, for their cooperation with monitor sampling. We thank the producers who facilitated our visits to their properties so that we could investigate cases. We acknowledge the support of veterinarians: Drs David Randell and Graeme Lean of Hamilton, and Drs Colin Scrivener, Jane Quinn and Martin Combs from Charles Sturt University, Wagga Wagga, NSW and Dr Bruce Johnson, TAS DPIWE.

10. Bibliography

Combs MDA, Rendell D, Reed KFM, Mace WJ, Quinn JC (2012) Dehydration and electrolyte disturbances contribute to severity of symptoms associated with perennial ryegrass toxicosis in Australia – insights from the 2011 outbreak. *Australian Veterinary Journal - submitted 13.12.2011*

Gallagher RT, Hawkes AD, Steyn PS, Vleggaar R (1984) Tremorgenic neurotoxins from perennial ryegrass causing ryegrass staggers disorder of livestock: structure elucidation of lolitrem B. *Journal of the Chemical Society Chemical Communication,* 614-616.

Genstat Committee (2008) Genstat ® for Windows, 11th Edition. VSN International, Hertfordshire, UK.

Hiatt EE, Hill NS, Bouton JH, Stuedemann JA (1999) Tall fescue endophyte detection: commercial immunoblot test kit compared with microscopic analysis. *Crop Science* **39**, 796-799.

Lane GA (1999) Chemistry of endophytes: pattern and diversity. *In* Ryegrass endophyte: An essential New Zealand Symbiosis. (DR Woodfield and C Matthew, eds.) New Zealand Grassland Association, Grassland Research and Practice Series No.7: 85-94.

Lane GA, Tapper BA, Davies E (1999) Ergot alkaloids additional to ergovaline in endophyte-infected perennial ryegrass and tall fescue in New Zealand. *In* Ryegrass endophyte: An essential New Zealand Symbiosis. (DR Woodfield and C Matthew, eds.) New Zealand Grassland Association, Grassland Research and Practice Series No.7: 95-100.

Panaccione D (2005) Origins and significance of ergot alkaloid diversity in fungi. *FEMS Microbiology Letters*, **251**: 9-17

Reed KFM, Walsh JR Cross PA, McFarlane NM and Sprague M (2004) Ryegrass endophyte (*Neotyphodium lolii*) alkaloids and mineral concentrations in perennial ryegrass (*Lolium perenne*) from southwest Victorian pasture. *Australian Journal of Experimental Agriculture* **44**, 1185-1194

Reed KFM, Moore DD (2009) A preliminary survey of zearalenone and other mycotoxins in Australian silage and pasture. *Animal Production Science* **49** 696-703.

Reed KFM, Scrivener CJ, Rainsford KA, Walker LV (2005) *Neotyphodium* research and application in Australia. pp. 43-54. In: *Neotyphodium* in Cool Season Grasses (Editors: Roberts CA, West CP and Spiers DE) Blackwell, Ames, Iowa.

Reed KFM, Page SW, Lean I (editors) (2005) Perennial ryegrass toxicosis in Australia. Meat and Livestock Australia, North Sydney. pp 97.

Reed KFM, Cummins LJ, Moore DD, Clark AJ (2011a) Performance of Coopworth ewe lambs exposed to low levels of ryegrass endophyte (*Neotyphodium lolii*) alkaloids and supplemented with a mycotoxin deactivator. *Animal Production Science* **51**, 225-232.

Reed KFM, Nie ZN, Walker LV, Mace WJ, Clark SG (2011b) Pasture and weather characteristics associated with outbreaks of perennial ryegrass toxicosis in southern Australia. *Animal Production Science* **51**, 738-752.

Reed KFM, Nie ZN, Walker LV, Kearney G (2011c) Fluctuations in the concentration of ergovaline and lolitrem B produced by the wild-type endophyte (*Neotyphodium lolii*) in perennial ryegrass pasture. *Animal Production Science* **51**, 1098-1108.

Reed KFM, Cummins LJ (2012) Letter to Ed. re Perennial ryegrass toxicosis. *The Australian Cattle Veterinarian* **63**:46.

Sackett D, Francis J (2006) The economic assessment of the impact of wild endophyte-infected perennial ryegrass (*Lolium perenne*) on the productivity of sheep and cattle and the profitability of Australian livestock enterprises. Report AHW.089, Meat and Livestock Australia. North Sydney

Sackett D, Holmes P, Abbott K, Jephcott S, Barber M (2006) Assessing the economic cost of endemic disease on the profitability of Australian beef cattle and sheep producers. Report AHW.087. Meat and Livestock Australia. North Sydney

Saikia S, Nicholson MJ, Young C, Parker EJ, Scott B (2008) The genetic basis for indole-diterpene chemical diversity in filamentous fungi. *Mycological Research*, **112**: 184-199

Shelby R and Flieger M (1997) Improved method of analysis for ergovaline in Tall Fescue by High Performance Liquid Chromatography. *Journal of Agricultural and Food Chemistry* **45**, 1797-1800

Shelby RA, Olsovska J, Havlicek V, Flieger M (1997) Analysis of ergot alkaloids in endophyte - infected tall fescue by liquid chromatography/electrospray ionization mass spectroscopy. *Journal of Agricultural and Food Chemistry* **45,** 4674-79.

Spiering MJ, Davies E, Tapper BA, Schmid J, Lane GA (2002) Simplified extraction of ergovaline and peramine for analysis of tissue distribution in endophyte-infected grass tillers. *Journal of Agricultural and Food Chemistry* **50**, 5856-5862.

Spiering MJ, Lane GA, Christensen MJ, Schmid J (2005) Distribution of the fungal endophyte *Neotyphodium lolii* is not a major determinant of the distribution of fungal alkaloids in *Lolium perenne* plants. *Phytochemistry* **66**, 195-202.

11. Photographs

Fig.1. PRGT case, Hamilton, 14.1.11

Fig. 2, Camperdown, 5.12.2010

Fig. 3, Camperdown 19.1.11

Fig. 4, Dookie 15.12.2010

Fig. 5, Dookie, 17.12.2010

Fig. 6, Outtrim 12.1.2011

Fig. 7, Wirra – 21.2.2011

Fig. 8, Cooroona - 9.3.2011

Fig. 9, Redwood - 15.3.2011

Fig. 10, Dookie – 28.4.2011

Fig. 11, PRGT sheep pastures at Woodside, Gippsland, 17.2.2012:

Fig. 12, Woodside, 17.2.2012

Fig. 13, Woodside, 17.2.2012

Fig. 14, Young steers, Birregurra, 7.3.2012

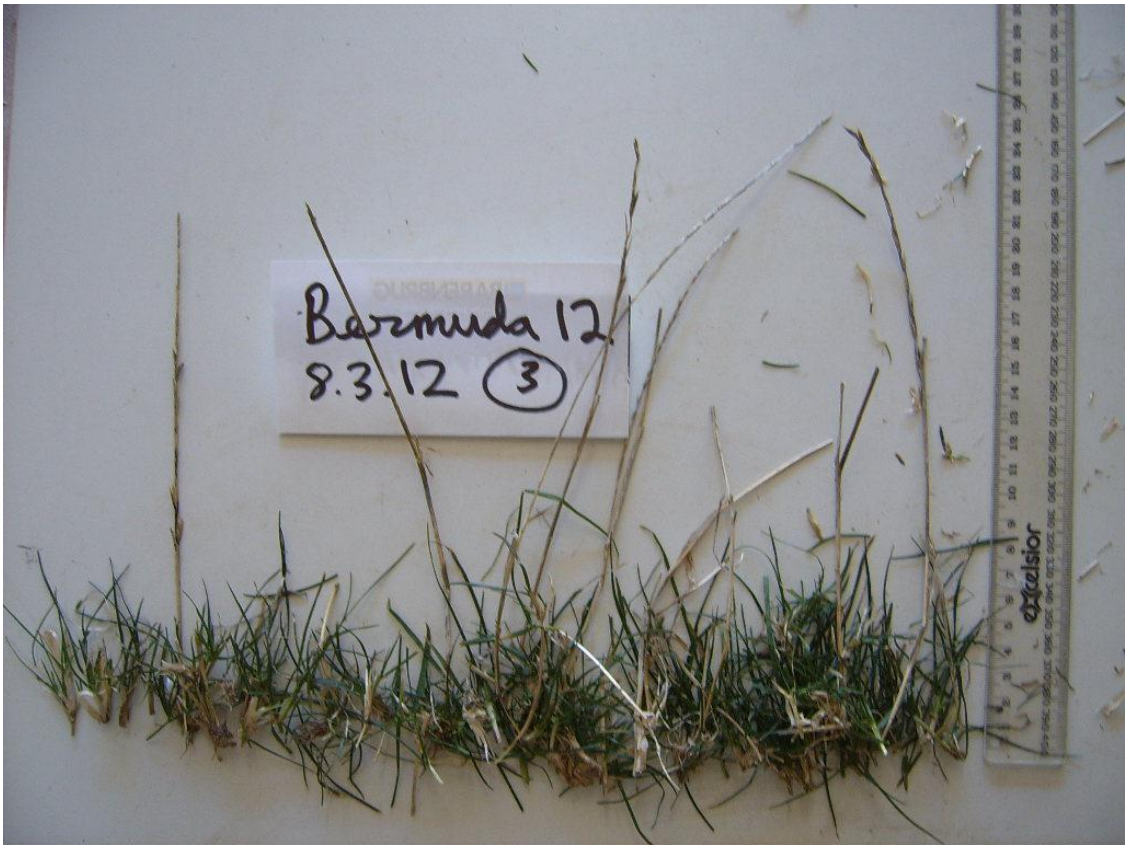

Fig. 15, PRGT pasture Hawkesdale, SW VIC, 8.3.2012

Fig. 16, PRGT pasture, Cressy, TAS, 29.3.2012

Fig. 17, PRGT pasture, Cressy, TAS, 29.3.2012