final report

Project code: B.AHE.0233

Prepared by: Jane Quinn, Martin Combs and Scott Edwards

Charles Sturt University

Date published: 31 August 2017

PUBLISHED BY
Meat & Livestock Australia Limited
PO Box 1961
NORTH SYDNEY NSW 2059

Treatment of clinical signs of perennial rye grass toxicosis in sheep

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

Abstract

Perennial Rye Grass Toxicosis (PRGT) causes significant mortalities in sheep during severe outbreaks and subclinical losses estimated to be in the region of 63M p/a. PRGT is caused by ingestion of a mixture of toxins present in *Neotyphodium lolii* infested perennial rye grass. The movement disorder 'Rye Grass Staggers' is the key clinical sign identifying high levels of the toxin lolitrem B being present in the pasture, a tremorgenic compound that causes the neurological signs, reaches levels sufficient to cause neurological effects. Alleviating the effects of PRGT in grazing livestock is of significant interest to producers and their economic partners alike; a therapeutic intervention effective in the most severe recumbent cases will increase survivability for animals during severe PRGT outbreaks. To identify a potential therapeutic agent a controlled clinical trial was undertaken in which clinical signs of 'Rye Grass Staggers' was induced in male lambs which were then treated with the anti-epileptic drug potassium bromide using one of two modalities. Animals receiving a single dose of potassium bromide showed significant improvement in their ability to maintain normal movement. Animals receiving a prophylactic dose over 22 days also showed improved mobility. This work suggests potassium bromide to be an effective treatment for Rye Grass Staggers, but more work is needed to confirm the correct application for prophylaxis.

Executive summary

Plant toxicoses cause major problems for cattle and sheep producers in Australia and result in significant economic loss in production animal industries annually(Sackett D 2006). Perennial Rye Grass Toxicosis (PRGT) is caused by a variety of toxins produced by the fungal endophyte, *Neotyphodium lolii*. When lolitrem B, a potent tremorgenic neurotoxin, reaches critical levels in pasture, a clinical syndrome, which consists of mild to severe neurological signs, known as 'Rye Grass Staggers' presents in affected flocks and herds. This occurs in animals grazing on endophyte-infested perennial rye grass under certain environmental conditions (Gallagher, White et al. 1981, Gallagher, Campbell et al. 1982, Gallagher, Hawkes et al. 1984). Although other alkaloid toxins are also implicated in the severity of presentation of PRGT in Australia, the extent to which these alkaloids play a role in the disease remains to be quantified. Currently there are no effective on-farm treatments for PRGT. As PRGT causes significant losses to producers in years with severe outbreaks(Sackett D 2006) alleviating the effects of PRGT in grazing livestock is of significant interest to producers and their economic partners alike.

In severe outbreaks, morbidity and mortality rates can be very high, with significant numbers of animals falling into permanent recumbencey or dying by misadventure, resulting in large production losses. Milder outbreaks still present with some acute mortalities although lost production due to altered management schedules, failure to present to market or poor weight gain and loss of condition are also significant in terms of economic cost. As such, producers managing PRGT outbreaks in Australia need to be able to prevent the intoxication and to treat clinical cases in order to reduce production losses. This will also improve animal welfare during PRGT outbreaks.

We evaluated potassium bromide as a treatment for Rye Grass Staggers in sheep using two delivery methods: 1) a single dose for treatment of acute staggers and 2) a prophylactic application delivered prior to onset of neurological signs.

On entry to the trial all animals were given a full clinical examination including full neurological examination which included proprioceptive testing, pupillary light reflex, menace reflex, eye position and movement and general cranial nerve function tests. Venous bloods samples were taken for clinical pathology, urine for specific gravity (USG) and electromyography for determination of normal / abnormal muscle activity. Body weight was recorded and heart rate, respiration rate and rectal temperature were recorded daily throughout the period of the trial.

Animals were exposed to a controlled diet containing 0.16mg/kg BW lolitrem B toxin rising to 0.27mg/kg BW after 21 days exposure to toxic feed. Toxin was delivered as perennial rye grass seed (GA66 AR98, Grasslanz Technology Ltd, NZ) containing very high levels of lolitrem B toxin (11ppm DM) as a component of the animal's feed intake.

Five treatment groups were established: a control group fed no toxic feed; a positive control group fed toxic feed but given no treatment; an acute treatment group fed toxic feed and administered a single dose of potassium bromide on first day of falling; a prophylactic treatment group fed both potassium bromide daily plus toxic feed for 22 days and a treatment only group given no toxic feed but delivered the prophylactic dose of potassium bromide.

Clinical signs were observed in all three toxic feed groups. The first observable signs of lolitrem B toxicity were a wide based stance, fine tremor of the head and neck and ventral eye deviation (strabismus). Intoxicated animals also exhibited a heightened state of nervousness showing increased reactivity to noise, touch or movement. Intention tremor became increasingly marked as toxicoses proceeded. After approximately 10 days, Type I movement disorder was noted in both positive control and acute treatment animals; being defined as an impaired alternating movement (dysdiadochokinesia), bunny-hopping gait and failure to maintain an appropriate direction. This progressed over a period of days to Type 2 movement disorder (Combs, Rendell et al. 2014); defined by increasing rigidity of limbs on forced movement resulting finally in tonic fore- and hindlimb rigidity with the animal collapsing into sternal or lateral recumbency (Combs, Rendell et al. 2014). Type 2 animals were able to recover and regain standing after a short period of time but would commonly fall if encouraged to move again. Identification of Type 2 movement disorder and day of falling was determined to be the date for entry to treatment for the acute treatment group. Time to falling was recorded for all animals and ranged from immediate upon encouragement to move to approximately 90 seconds after encouragement to move. On neurological examination, intoxicated animals were found to have a normal pupillary light reflex but reduced or absent menace response with involuntary eye movements (nystagmus) of increased amplitude.

On day of falling, acute treatment animals were given an oral dose of 300mg/kg BW of potassium bromide. Movement, neurological signs and general clinical signs were examined 24 and 48 hours after treatment at which point animals were euthanased for full post mortem. Treatment with potassium bromide was observed to significantly improve time to falling in intoxicated animals with most (8/9) animals failing to fall when driven to movement for a continuous period of 5 minutes. Positive control animals, which had not been treated with potassium bromide, showed no improvement over the same time period (7/7 falling).

Clinical pathological analysis of serum from all treatment groups showed no significant difference in any biochemical markers associated with liver function. Renal function was observed to be mildly compromised in toxin only and acute treatment groups with mild elevations in creatinine and urea observed but not outside normal ranges for our cohort. USG was also marginally increased compared to reference ranges. Although not considered to be clinically significant in these animals it does demonstrate increasing fluid losses and/ or decreasing fluid intake in intoxicated animals; this is likely to be of greater clinical importance in the field where animals can be under heat and nutritional stress.

Early pathological lesions were noted in the brain of intoxicated animals; these included small numbers of spheroids located in the granule cell layer, mainly in the lateral cerebellum, as well as pyknotic granule cell nuclei and occasional vacuolated Purkinje neurons. These changes represent the earliest lesions of PRGT reported.

Animals in the potassium bromide prophylactic group also showed improved movement with treatment with only one in this treatment group progressing to falling in the designated 22 days of treatment compared to 5 animals in Groups 2 and 3 over the same timeframe. Animals in this group showed other neurological signs similar to their toxin only counterparts.

The data presented from this study confirm potassium bromide (KBr) as an effective therapy for neurological PRGT, or “Rye Grass Staggers”, with indication of usefulness as a prophylactic treatment. Further trials are needed to refine dosing for prophylactic application. Progression to APVMA registration of potassium bromide as a remedy for sheep is advised.

Table of contents

1 Background

Perennial rye grass toxicosis (PRGT) affects livestock grazing on perennial ryegrass infected with the endophytic fungi *Neotyphodium lolli* which produces the potent indole diterpenoid toxin lolitrem B and the ergot alkaloid ergovaline (Gallagher, Campbell et al. 1982, Cheeke 1995). Depending on the relative proportions of the toxins produced by the particular endophyte, clinical signs of PRGT include abnormal behaviour, ataxia ('staggers'), ill thrift and gastrointestinal dysfunction (scours) which have been linked to moderate or high levels of lolitrem B toxin in pastures (Cunningham 1959, Reed, Vaughan et al. 2010, Reed, Nie et al. 2011). Clinical signs range in severity from mild gait abnormalities and failure to thrive to severe seizures, lateral recumbency and death (Finnie, Windsor et al. 2011). Mild outbreaks result mainly in subclinical production losses, management and animal welfare issues for affected producers whilst severe outbreaks can involve significant stock losses (Sackett D 2006). Clinical presentation is usually highly variable with season, breed, sex, age and production status all affecting severity of clinical signs in the flock or herd (Finnie, Windsor et al. 2011, Reed, Nie et al. 2011). A particular feature of PRGT in Australia is the occasional occurrence of large scale sheep losses suggesting other factors are influencing mortality rates here compared to other PRGT risk zones.

Currently no effective treatment exists for PRGT in livestock. However, a number of approaches have been evaluated but, to date, no effective therapeutic suitable for on-farm delivery has been identified.

1.1 Project objectives

The objectives of this project were:

- To test the efficacy of KBr for the prevention and treatment of the clinical signs of PRGT.
- To test two treatment modalities: a single oral dose as a acute treatment after onset of clinical signs, or as a daily prophylactic dose during the toxin loading phase of the trial.

2 Methodology

This study was undertaken with approval from Charles Sturt University Animal Care and Ethics Committee (protocol 13/033).

2.1 Animals

Animals were White Sussex x Merino first cross male lambs of between 10-12 months of age (n=45, live weight 36.1 ± 3.02 kg) sourced from a single producer. They had been maintained under normal husbandry conditions grazing mixed lucerne and wheat stubble pastures prior to entry to the trial. No animals used exhibited any obvious pre-existing pathology. Animals were housed for the duration of the trial in individual pens at the NSW DPI Animal Nutrition Facility, Charles Sturt University, Wagga Wagga. Animals were allowed to acclimatise to the animal house for 7 days prior to entry to the trial. During this period they were fed a restricted diet of lucerne chaff, approximately 2.5% live weight, and had access to water *ad libitum*.

2.2 Toxic feed

To generate a feed containing toxic levels of lolitrem B without confounding high levels of ergovalline, a novel endophyte-infested perennial rye grass seed (Ga66 AR98, Grasslanz technology Ltd, New Zealand) was sourced. Toxin analysis by LCMS was carried out by AgResearch, New

Zealand, which showed the seed to contain 11.1mg / Kg DM lolitrem B toxin. No ergovalline was detected on quantisation.

On entry to the trial, all animals receiving toxic feed were exposed to a diet containing experimental ryegrass seed with a final lolitrem B concentration of 0.08mg / KG LW, lucerne chaff and molasses 30% w/v for 3 days. After this induction period, the toxin content of the feed was increased to 0.16mg / kg LW for the duration of the trial. For any animal that had not showed Type 2 movement disorder at 21 days, the dose was increased to 0.27 mg/kg LW until falling. Feed analysis was carried out by the DPI NSW Feed Quality Testing Laboratory, Wagga Wagga, NSW and the DPI Diagnostic and Analytical Services Environmental Laboratory, Wollongbar, NSW. Ryegrass seed constituted to up to 65% of available Feed on Offer (FOO) by dry weight contributing a metabolisable energy of 12.1Mj/Kg DM.

2.3 Physiological monitoring

At entry to the trial, all animals were subjected to a full clinical examination. Live weight was recorded. Venous blood and urine samples were collected for laboratory. Animals were also subjected to gait analysis, neurological examination including proprioceptive testing, cranial nerve examination and papillary light and menace reflexes. Electromyography (EMG) of tricep and neck muscles was performed. Gait analysis (see below) was repeated at three day intervals until onset of significant clinical signs and / or gait abnormality at which point animals were subjected to gait analysis daily. Gait analysis was recorded on video each occasion.

General clinical signs were also noted on a daily basis; these included observations of nervousness or agitation, changes in normal placement of body, limb or head position changes in faecal consistency, feeding behaviour or water consumption, observable tremor of the body or head, eye position (strabismus) and movement (physiological/ pathological nystagmus), locomotor disturbances, or any other clinical changes worthy of note. Feed and water intake was monitored daily for the duration of the trial. Heart rate, respiration rate at rest and rectal temperature were also monitored daily.

2.4 Treatments

2.4.1 Treatment rates for potassium bromide

The anticonvulsant range of bromide in monogastric species is 0.8-2.0mg/ml (Podell and Fenner 1993). Concentrations of Br which will prevent, attenuate or abolish PRGT tremor in ovines are currently unknown. As such, the lower bound of the monogastric anticonvulsant range was initially used as the target blood concentration in sheep. Using the equation Loading Dose (LD) = *V* * target concentration , and factoring in the 92% oral bioavailability of bromide in sheep, a LD of 340mg/kg was obtained as an acute treatment.
To determine potassium bromide dosing for prophylaxis, a small pre-trial was performed in which 3 sheep were delivered an acute oral dose of 500mg/kg in a single oral bolus. Palatability was problematic as the concentration of bromide in solution was high (400g/L) and mild sedation was observed, presumably a function of high peak bromide concentration. In light of this finding, and to mitigate these effects, it was decided that subsequent prophylactic sheep were to receive a split loading dose of 300mg/kg on day 1, 120mg/kg (100mg LD + 20mg/kg daily maintenance dose) on day 2, 120mg/kg on day 3 and 20mg/kg daily for the duration of the trial. On the basis of this analysis, and knowledge that bromide as an ionic salt would be difficult to ingest orally in large quantity, an acute treatment dose of 300mg/kg LW was used for the trial. Both prophylactic and acute treatment doses were envisaged to give serum concentrations of 750-1000mg/ml for the duration of the initial weeks of the trial.

2.4.2 Establishment of treatment groups

There were five treatment groups:

Group 1 - Negative Control: lucerne chaff only;

Group 2 - Positive Control: lucerne chaff containing 0.16 mg/kg LW lolitrem B;

Group 3 - Acute KBr treatment: lucerne chaff containing 0.16mg/kg LW lolitrem B, treated orally with 300mg/Kg bromide (Sigma Aldrich) on day of falling;

Group 4 - Prophylactic KBr treatment: lucerne chaff containing 0.16mg/kg LW lolitrem B, treated orally with a prophylactic therapeutic dose of potassium bromide with a loading dose give over 72 hours on entry to the trial with a continued maintenance dose administered orally daily (see below);

Group 5 - Prophylactic KBr treatment control: lucerne chaff only, treated orally with prophylactic potassium bromide as described below.

Prophylactic treatment sheep (Groups 4 & 5) received a split loading dose of 300mg/kg on day 1 of the trial, 120mg/kg (100mg LD + 20mg/kg daily maintenance dose) on day 2, 120mg/kg on day 3 and 20mg/kg daily thereafter. Acute therapy sheep were dosed with a single dose of 300mg/kg on day of falling.

Animals entered the trial in cohorts of 5, each cohort containing one animal from each treatment group with entry occurring over 3 consecutive days such that groups of 15 animals undertook the trial together. Three sets of 15 animals were used for this study (n = 45) with each treatment group containing 9 animals. Animals were maintained on the treatments as described above for the duration of the trial period.

End of the trial was defined for Groups 2 and 3 as date of falling. Group 1 animals entered the end of trial protocol with their Group 2 counterparts. Group 4 and 5 animals entered the end of trial protocol after 22 days. This end of trial protocol consisted of the following analysis:

- On Day1: animals were subjected to a full clinical examination, gait analysis, urine and blood collection, neurological examination and EMG.
- On Day 2: further gait analysis was recorded.
- On Day 3: animals were again subjected to full clinical examination, gait analysis, urine and blood collection, neurological examination and EMG as well as live weight and body condition score recorded prior to necropsy.

2.5 Electromyography

EMGs were recorded on entry to the trial, entry to the end of trial (Day 1) and date of post mortem (Day 3) using Powerlab™ (ADInstruments, Castle Hill, Australia) and data recorded using LabChart™ software (ADInstruments, Australia). In preparation, the fleece was shorn from an area over the triceps muscle and along brachiocephalicus muscle of the neck (see Figure 1). EMG electrodes were attached to the skin using SuperGlue™. Muscle activity was recorded for a minimum of three minutes with the animal standing at rest with a band pass filter set at 100Hz. Three minute EMG recordings were taken using Ag-AgCl surface electrodes over the triceps muscle on to entry to the trial, on first day of falling (Day 1) and 48hours post treatment (Day 3). Data analysis was achieved

by application of a Fast-Fourier transformation for statistical comparison. Area under the curve was then calculated at frequencies between 5 and 30Hz to estimate tremor intensity

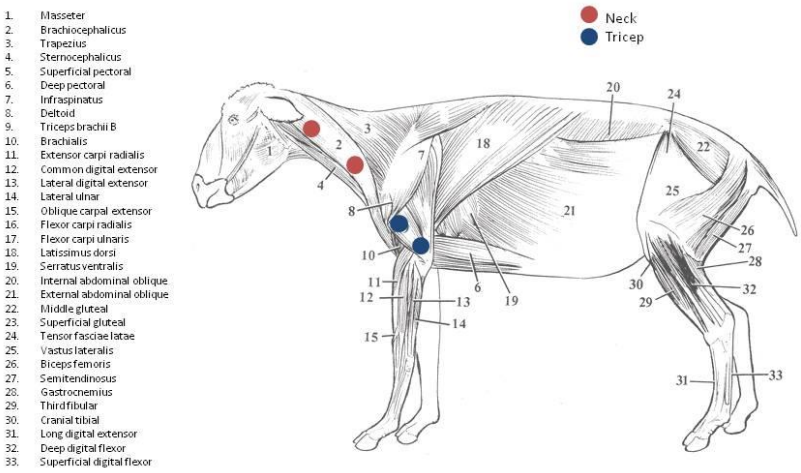

Figure 1. Position of placement of electrodes for electromyography. Adapted from http://www.infovets.com/books/smrm/A/A030.htm

2.6 Gait analysis

Gait analysis was performed on entry to the trial and at designated time points throughout the trial as described above. To achieve this, animals were moved from their individual pens to an external yard in their cohort groups (5 animals, one from each treatment). Animals were then encouraged to move at a run, initially in a group and then individually, for a minimum of three minutes per animal whilst their movement was captured on video and gait observations recorded.

Once all animals had been analysed the whole cohort was returned to their individual pens. Gait abnormalities such as stumbling, falling or disorientation were noted, including observation of Type 1 or Type 2 gait changes as described in Combs et. al., (2014). Type I movement disorder is defined as an impaired alternating movement (dysdiadochokinesia), bunny-hopping gait and failure to maintain an appropriate direction. Type 2 movement disorder is defined by increasing rhythmic myoclonus with hyperextension of limb or occasionally mark hyperflexion on forced movement resulting finally with the animal collapsing into sternal or lateral recumbency (Combs, Rendell et al. 2014). Scores between those denoted above were considered as a gradation between the stated clinical observations. Analysis of gait was performed using the following scale in Table 1 and a composite score noted for each animal for each day during the end of trial protocol. A total score out of 30 was determined for each animal. The higher the score, the greater the locomotor disturbance. Time to falling was determined post trial by analysis of video material.

Table 1. Gait analysis, scale of clinical observations.

	Score		
Clinical observation	**1**	**3**	**5**
Dysdiadochokinesis	Mild disunited fore / hind limb coordination on acceleration	Changes in forelimb / hindlimb coordination such as pacing at moderate pace or gait	Continuous bunny-hopping gait
Rhythmic Myoclonus	Mild hyperextension/ hyperflexion (hind > fore)	Increased severity, significant forelimb involvement with limb extension and/ or inability to extend limb	Severe – all four limbs involved with characteristic arching of back.
Directional movement	Reduce ability to change direction	Frequently moves in abnormal direction (i.e. near misses with stationary objects)	Unable to control or maintain direction (frequently hits stationary objects)
Ataxia	Mild - wide based gait only	Body rolling, wide based gait	Body rolling and frequent limb crossing
Failure to maintain ambulation	Stumbling without falling on rapid movement	Frequent stumbling without falling	Falling
Clonic seizures			Seizures

2.7 Laboratory analysis

All laboratory analysis was performed by staff of the Veterinary Diagnostic Laboratory (Charles Sturt University, Wagga Wagga, NSW Australia). Venous blood samples collected for biochemical analysis and haematocrit (PCV), and urine for USG, at three specific time points during the experiment for all animals: 1) on entry to the trial; 2) on date of entry to the end of trial protocol and, 3) on day of necropsy. Additional venous blood samples were occasionally collected for analysis of serum bromide (see below).

For venous sampling, blood was collected from either the right or left jugular vein as determined by convenience and sampler preference. Needle hubs and sterile, single-use 21G needles and 10 mL Vacutainer® tubes were used for collection. Two Vacutainer® tube types were used for each animal: one containing a clot activator for serum collection, the other contained ethylenediamine tetra-acetic acid (EDTA) to prevent clotting. Tubes were inverted repetitively immediately after collection prior to storage on ice for transport to the laboratory. EDTA tubes were kept chilled until processing. Serum blood tubes were allowed to clot for at least 30 minutes before serum separation by centrifugation at 1000rpm for 10 minutes. All samples were transported and processed within 60 minutes of collection.

Urine samples were collected by attachment of a clean plastic bag using SuperGlue™ to the fleece on either side of the preputial opening. The bag was removed as soon as urine collection had been achieved and samples transferred to a clean plastic container. Samples were stored at 4^{o}C prior to

removal to the laboratory for processing. Urine specific gravity (USG) was determined using a refractometer (VQ5600 refractometer, VetQuip, Australia).

Haematological and biochemical analysis was performed by the laboratory within 60 minutes of blood collection. Haematological analytes were measured using a CellDyn 3700 Haematology System (Abbott Diagnostics, Abbott Park, Illinois, USA). Biochemical analysis was performed using a Konelab 30i clinical chemistry analyser (Thermo Electro Corp., Vantaa, Finland), using reagents from Thermo Scientific. See Appendix 1 for a full list of haematological analytes reported.

2.8 Necropsy

Necropsies were performed at the Veterinary Diagnostic Laboratory, Charles Sturt University, Wagga Wagga. A full clinical examination was performed prior to euthanasia. At euthanasia, 100 I.U. heparin (Provet Riverina Pty Ltd) was injected into the jugular vein, followed shortly by Lethabarb (20ml/40kg LW). Immediately after euthanasia, the ventral articulation of C1 was exposed and cerebrospinal fluid (CSF) collected using a 23g needle. The head was then removed and subjected to perfusion fixation. Briefly, the carotid arteries were exposed and a small catheter inserted and ligated to secure the perfusion lines. Two litres of 0.1% phosphate buffered saline (PBS) containing 3000 I.U of heparin /L was then slowly perfused via the ligated vessels at a pressure of 90mm Hg using a Microgon peristaltic Pump (Microgon, Laguna Hills, California). Once complete this was replaced with 4% paraformaldehyde (Sigma-Alrich) in 0.1% PBS and a further two litres allowed to perfuse slowly through the tissues of the head. Once complete, catheters were removed and the whole head placed at 4^{o}C overnight to complete fixation. Twenty-four hours post perfusion fixation the brain was removed from the skull and placed in 10% formal saline for storage until dissection for processing to wax sections.

Concurrent to fixation perfusion of the head, a routine ovine necropsy was performed and all general tissues taken for routine histopathology. These were heart, lung, oesophagus and trachea, liver, spleen, gut (rumen, abomasum, ileocaecal junction, ileum, caecum and colon), skeletal muscle (gluteal, tricep and diaphram), kidney, pancreas, thymus, ileal and cranial lymph nodes as well as tissues from any gross abnormalities observed at time post necropsy. Other samples: subcutaneous fat, renal fat, gluteal muscle, kidney, faeces and rumen contents were collected and stored at -80^{o}C for future toxicological analysis. Samples for routine histopathology were fixed in 10% formal saline for a minimum of 48 hours before processing to wax using an automated wax embedder (Shandon Excelsior ES Tissue Processor, Thermo Fisher Scientific).

For histopathology, sections of brain and spinal cord included the following regions: mid cerebellar peduncle; mid and lateral sections of cerebellum; obex; occipital cortex; rostral colliculi; basal ganglia; frontal cortex; temporal cortex; thalamus; hippocampus; internal capsule; cervical, thoracic and lumbar spinal cord and pituitary. Other tissues examined for routine histopathology were kidney; lung; ileum; heart; liver including, gall bladder and bile duct; diaphragm; rumen; and colon.
All sections were cut at 5□m and stained with Haematoxylin and Eosin (H&E) using an automated staining system (Shandon Varistain Gemini ES Slide Stainer, Thermo Fisher Scientific).

2.9 Analysis of serum and cerebrospinal bromide concentrations

Serum Bromide concentrations were determined by colorimetric spectrophotometry as previously described (Tietz 1976), with some modification. Briefly, 0.5 mL of serum was added to 4.5 mL of 10% trichloroacetic acid (Sigma-Aldrich) in a 10 mL centrifuge tube, vortexed, then centrifuged for 15 min at 2000 g. 2.5 mL of supernatant was then mixed with 0.25 mL of 0.5% Au_2Cl_6 (Sigma-Aldrich) and left to stand for 30 min. Absorbance was measured with a spectrophotometer at 440 nm. The

standard curve was linear in the range of 25 µg/mL to 5000 µg/mL, R^2= 0.9992. The lower limit of quantification (LOQ) was 25 µg/mL.

2.10 Statistical analysis

Reference intervals were defined as the interval containing the central 95% of data obtained for each analyte after the exclusion of outliers. Outliers were excluded based on the method proposed by Dixon (Dixon 1953), and modified by Reed, Henry & Mason (Reed 1971). Distributions of the outlier-excluded values were tested for normality using the Kolmogorov-Smirnov test using IBM SPSS™, Version 20.0.0. A Kolmogorov-Smirnov value > 0.050 was the criterion for describing the data as a normal distribution. For analysis of biochemical data, a simple liner regression was used with Kendall's correlation analysis applied to for non-parametric data sets.

3 Results

3.1 Establishment of an experimental model of 'Rye Grass Staggers' in sheep

Clinical signs attributable to PRGT in field cases vary in their description. Generally the syndrome has been characterised by neurological changes such as head shaking, ill-coordination, staggering and collapse(Cheeke 1995) with spinovestibular cerebellar signs noted including eye deviation (Mayhew 2009). The movement disorder associated with 'ryegrass staggers' represents a specific sequence of dyskinesis which has been only recently fully characterised (Combs, Rendell et al. 2014). To determine establishment of a comparable clinical syndrome to that observed in field cases of perennial rye grass toxicosis, detailed neurological observations and gait analysis were performed systematically throughout the trial to define the earliest observable neurological signs as well as progression from no movement disorder, to Type 1 and Type 2 gait changes as defined by Combs et al., (2014) (see Section 2.6).

3.2 Time to onset and clinical signs associated with experimental lolitrem B toxicosis

Neurological signs of lolitrem B intoxication followed a clear progression. The first observable clinical signs were a fine tremor of the head and neck, ataxia presenting as an alteration in stance or limb placement at rest which generally coincided with onset of Type 1 gait changes (see Table 2). Ventral strabismus was also observed in a proportion of intoxicated animals affecting 9/9 animals in Group 2, 7/9 in Group 3 and 6 (animals in Group 4. Type 2 gait changes followed Type 1 approximately 9 days later with a range of between 1 and 27 days (Table 2). Observations of Type 2 gait changes were usually coincident within 4 days of falling. Menace reflex was either lost or reduced in lolitrem B intoxicated animals but their pupillary light reflex was normal.

This pattern represented a clear progression of disease:

- **Early clinical signs:** mild tremor of the head and neck, ventral strabismus, abnormal body stance at rest, increased reactivity;
- **Intermediate clinical signs:** Type 1 movement disorder, stumbling usually of forelimbs, gross limb and / or body tremor, inappropriate decision making regarding direction, increased reactivity to noise movement, ventral strabismus;

- **Advanced clinical signs:** Type 2 movement disorder, inability to follow a direction of movement for an extended period, stumbling and falling, collision with objects, collapse into sterna or lateral recumbency, generalise myoclonus, limb extension and opisthotonos, significant altered body stance at rest, heightened reactivity to noise / movement, loss of menace reflex.

Histopathological changes at necropsy mimicked those observed in field cases (Parton K 2006 , Combs, Rendell et al. 2014) with some additional features (see Figure 2). Lesions were restricted to the cerebellum and consisted of three specific histological features:
1) spheroids (axonal swellings) located in the granule cell layer;
2) presence of pyknotic granule cell neurons in the granular layer and less frequently,
3) interneuronal vacuolation of Purkinje Neuron cell bodies.

Spheroids were observed to be present in the molecular layer of the cerebellum but these may be incidental to those observed in the granular layer and / or represent a background lesion.

Table 2. Clinical signs associated with ingestion of lolitrem B-containing toxic feed and treatment with either acute or prophylactic potassium bromide (KBr).

	Mean number of days to onset (+/- s.e.m.) ***Number of animals affected***					
Group	**First clinical signs**	**Fine tremor**	**Altered stance**	**Major body clonus**	**Type 1 gait**	**Falling**
Group 1 – Control	0 *0/8*	0 *0/8*	0 *0/8*	0 *0/8*	0 *0/8*	0 *0/8*
Group 2 – Toxin only	7.56(+/-2.06) *9/9*	8.22(+/-2.58) *9/9*	11.78(+/-2.58) *9/9*	17.33(+/-3.51) *3/9*	10.22(+/-4.46) *9/9*	25.75(+/-5.28) *8/9*
Group 3 – Toxin KBr Acute Txt	8.56(+/-1.94) *9/9*	12.43(+/-4.82) *7/9*	10.22(+/-2.10) *9/9*	11.67(+/-2.88) *3/9*	8.99(+/-1.26) *9/9*	27.56(+/-9.91) *9/9*
Group 4 – Toxin KBr Proph Txt	6.33(+/-1.73) *9/9* [a]	12.14(+/-4.45) *7/9*	8.33(+/-1.22) *9/9*	10.67(+/-2.65) *5/9*	12.44(+/-4.71) *9/9*	22.0(+/-0.0) *1/9*
Group 5 – KBr Proph Txt	10.75(+/-4.46) *8/9* [b]	11.00(+/-0.0) *1/9*	11.0(+/-4.69) *4/9*	0 *0/9*	0 *0/9*	0 *0/9*

[a]Includes 4/9 animals showing evidence of mild anxiolysis
[b]Includes 5/9 animals showing evidence of mild anxiolysis

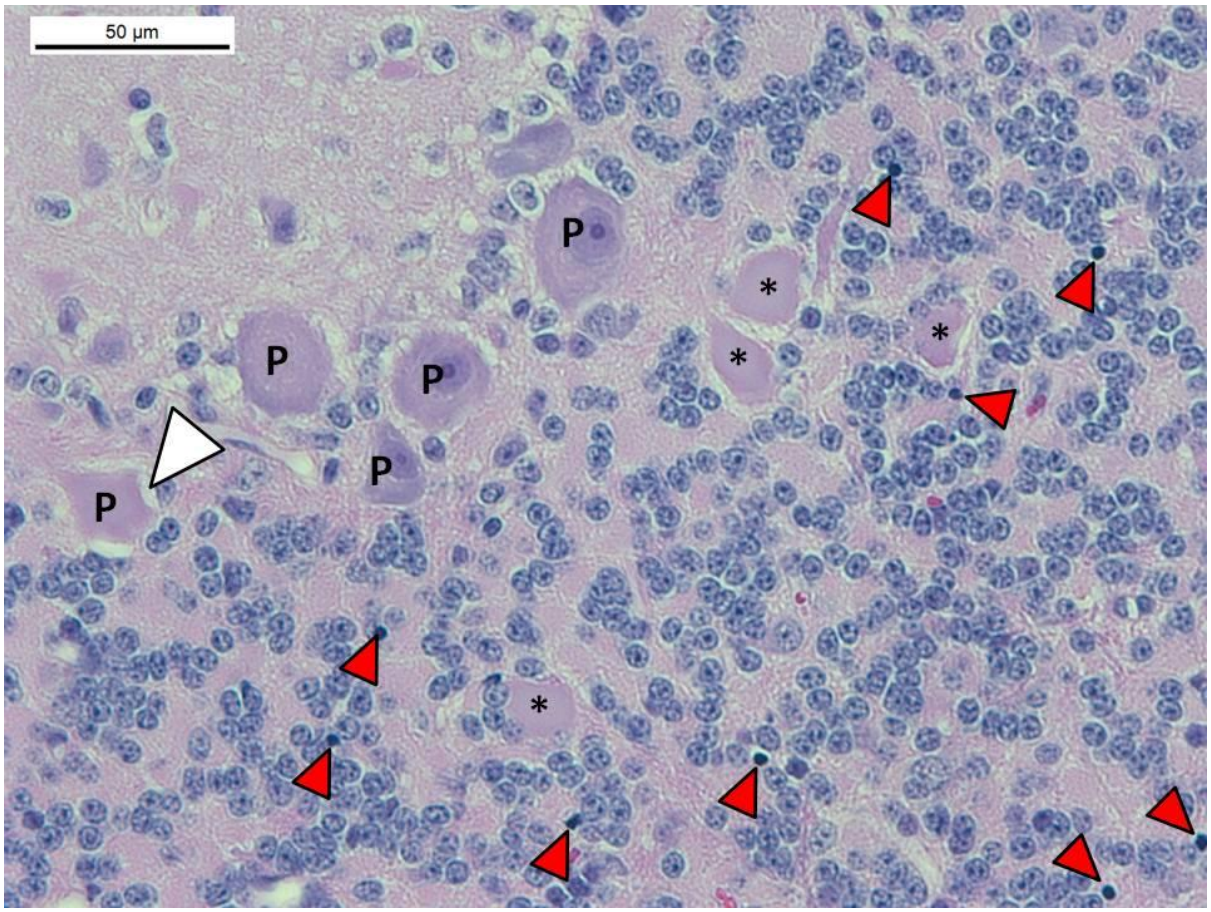

Figure 2. Histological lesions observed in the cerebellum of animals intoxicated with lolitrem B. Three clear histopatholgical lesions are noted: spheroids (*) and pyknotic nuclei (red arrowheads) present in the granular later and vacuolation within Purkinje neurons (white arrow head).

Intraneuronal vacuolation of Purkinje neurons is likely to represent the earliest neuropathologal changes in this cell type. Formation of spheroids, in the absence of other notable pathology, is commonly reported as a 'diagnostic' lesion in field cases of PRGT. One animal in the control group (Group 1) presented with neurological lesions on histopathological suggestive of an underlying neuropathy. This animal was excluded from further analysis on this basis.

Lesions were observed in the highest number in those animals exposed to lolitrem B toxin only (Group 2, Table 2) with 7/9 animals presenting in the granular layer with spheroids and 9/9 exhibiting pyknotic cell bodies. A similar pattern was observed in the other 2 toxin treated groups (Groups 3 & 4, Table 3). In all toxin treated groups, spheroids and pyknotic cell bodies in the granular layer were the most common finding. A lower incidence of spheroids in the molecular layer was noted, the incidence included both the prophylactic treatment only group, (Group 5) and control group (Group 1) suggesting that this may be an incidental finding in these cases (see Table 3).

Pyknotic nuclei, indicative of cell death in the granular layer of the cerebellum have not been previously reported in field cases of PRGT. This suggests that these are acute lesions or have been overlooked in previous investigations due to lack or 'normal' control animals for comparison. No other lesions were found in any other area of the brain examined.

Table 3. Acute histopathological lesions of the cerebellum associated with ingestion of lolitrem B-containing toxic feed and treatment with either acute or prophylactic potassium bromide (KBr). Abbreviation: PN, Purkinje neuron. Key to groups: 1: no toxin, no treatment control; 2: toxin only; 3: toxin plus acute treatment; 4: toxin plus prophylactic treatment; 5: prophylactic treatment only.

	Incidence			
Group	**Spheroids**		**Purkinje neuron vacuolation**	**Pyknotic cell bodies in granular layer**
	Molecular layer	**Granular layer**		
1	**1/8**	**0/8**	**1/8**	**0/8**
2	**1/9**	**7/9**	**5/9**	**9/9**
3	**0/9**	**7/9**	**2/9**	**5/9**
4	**3/9**	**6/9**	**1/9**	**9/9**
5	**3/9**	**1/9**	**0/9**	**2/9**

Incidental pathological findings, considered to be irrelevant to the experiment, included some systemic changes: notably mild lymphocytic cholangeohepatitis (animals 7 & 9, Group 4; animals 8 & 9, Group 5); mild lymphocytic hepatitis (animals 2 & 4, Group 1; animal 1, Group 5); mild lymphycytic pyelitis (animal 2, Group 1; animal 3, Group 2; animal 3, Group 3 and animals 7 & 9 Group 5); mild interstitial nephritis (animal 3, Group 1; animals 5 & 6 Group 2; animal 5 , Group 5); mild tubular nephropathy (animal 9, group 5); bronchopneumonia and pleuritis (animal 7, Group 5); mild lymphocytic colitis (animal 7, Group 3) and lymphadenitis (animal 2, Group 5). Multifocal myositis and multifocal lymphycytic myocarditis was observed in all animals secondary to presence of sarcosysts. Again, these are suggested to be incidental background lesions identified due to the size of the cohort.

3.3 Treatment with a single acute dose of oral potassium bromide decreases severity of tremor, increases time to falling and improves gait in lolitrem B intoxicated animals

Animals that met the entry criteria (severe Type 2 gait abnormality and falling) were submitted to the end-of-trial protocol. This consisted of acute treatment with 300mg/kg potassium bromide orally; three days of testing, starting on date of falling, with necropsy on Day 3. Animals were subjected to the following tests on days 1 and 3: gait analysis, full neurological examination, venous blood and urine sampling and EMG. Only gait analysis was performed on day 2.

Bromide concentration in serum was monitored 6 hours, 24 hours and 48 hours after treatment. Similar to that observed in the pre-trial PK study, serum concentrations rose sharply in the first 6 hours after administration (mean serum concentration 6 hours: 899.43□g/ml +/- 56.44, n = 3), falling slightly over the 48 hour treatment period to a final mean serum concentration of 804.34□g/ml +/- 24.26, (n = 6) after 48 hours. This analysis indicates that high levels of bioavailability of potassium bromide from 6 hours post treatment (see Figure 3). This data is supported by PK studies performed prior to this trial in which high levels of bioavailability were also observed with serum bromide levels peaking at 6 hours post administration (Combs and Edwards, personal communication).

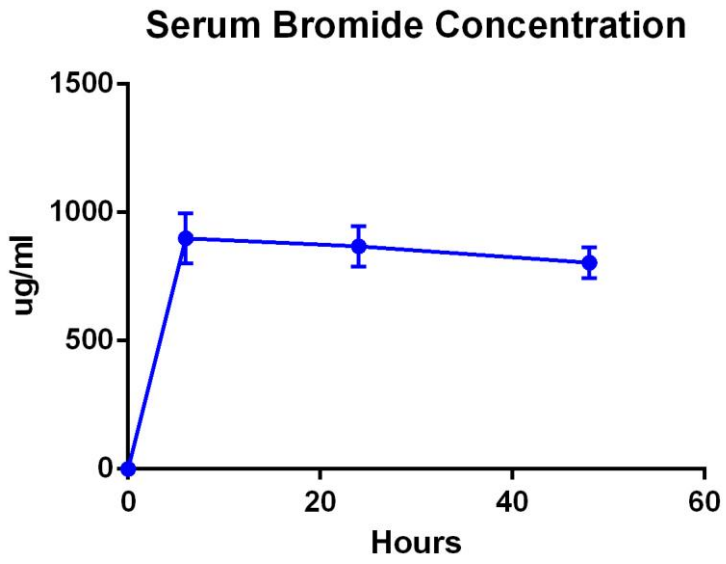

Figure 3. Serum concentrations in lolitrem B-intoxicated sheep treated with an acute oral dose of potassium bromide.

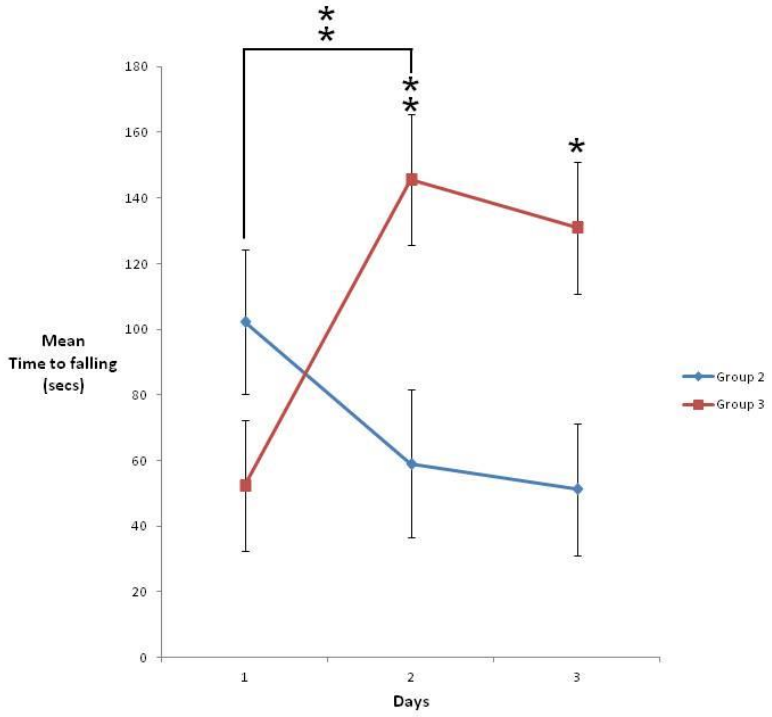

Figure 4. Time to falling in seconds of animals exposed to lolitrem B toxin only or toxin plus treatment with acute oral potassium bromide. Values shown for Day1 for Group 3 represent time to falling prior to treatment Group 2 (lolitrem B toxin only, n = 8) and Group 3 (lolitrem B toxin plus single acute treatment with potassium bromide, n = 9).

* Significant difference between pre- and post trial samples, p = <0.05

** Significant difference between pre- and post trial samples, p = <0.01

Animals treated with a single oral dose of bromide 300mg/KG LW showed significant extension in time to falling 24 hours after treatment (Group 2 day 1: 52.44 seconds ± 19.55 seconds; Day 2: 145.66 ± 22.07 seconds; students t test; p = 0.002, Figure 4). Although no significant difference was observed between days 2 and 3 within treatment groups, differences between groups on both days were highly significant (Group 2 Day 2: 145.66 ± 22.07 seconds; Group 3 Day 2: 59.0 ± 23.50 seconds; p = 0.003; Group 2 Day 3: 124.75 ± 22.06 seconds; Group 3 Day 3: 51.52 ± 20.23 seconds; p = 0.02, Figure 3). This data represents a significant increase in time to falling in Group 3 (toxin plus acute treatment) on days 2 and 3, compared to day 1, whilst a coincident reduction in time to falling is observed in untreated animals (Group 2, toxin only) (Figure 4). This data suggests improved coordination in the acute treatment group (Group 3) with a deterioration with increasing toxin load over the same timeframe in their untreated counterparts.

Gait analysis showed a similar trend with composite scores decreasing in the potassium bromide acute treatment group (Group 3) indicative of a return to normal gait characteristics on treatment, whilst scores increased over time in their untreated counterparts (see Figure 5). A significant reduction in composite gait score was observed between Day 1 (pre treatment) and Day 2 (24 hours post treatment) in Group 3 animals which were exposed to lolitrem B toxin and treated orally with 300mg/kg LW potassium bromide (p = 0.001, Figure 5). This is consistent with the improvement in maintenance of normal ambulation observed by increased time to falling post treatment (see Figure 4). Conversely, composite gait scores are observed to increase in untreated intoxicated animals (Group 2) over the same time course (p = 0.05) showing a deterioration in gait over time.

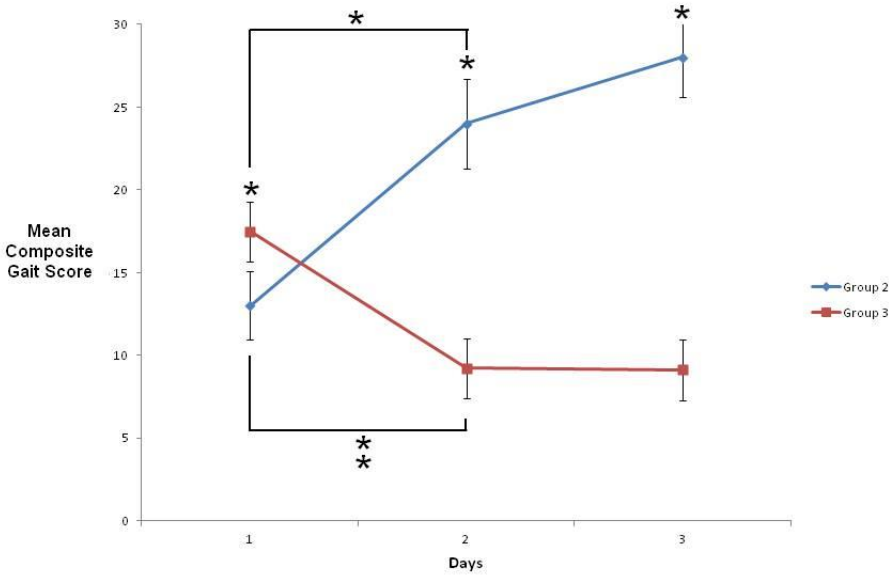

Figure 5. Mean composite gait score over time for animals exposed to lolitrem B toxin only or toxin plus treatment with acute oral potassium bromide.
* Significant difference between pre- and post trial samples, p = <0.05
** Significant difference between pre- and post trial samples, p = <0.01

Treatment with potassium bromide was also found to decrease tremor intensity in intoxicated animals. Both Group 2 and Group 3 animals showed significant increases in tremor intensity between pre-trial and Day 1 of testing (p = 0.002). Neither group showed a significant difference within groups between Days 1 and 3. However when Day 1: Day 3 tremor intensity ratios are compared between groups there is a significant difference (p = 0.01, see Figure 6). The median ratio for the Group 2 was 2.03 indicating an increasing intensity of tremor whereas in Group 3 the mean ration is 0.81 indicating a stabilisation of the tremor despite increasing intoxication over this time period (Figure 6).

Figure 6. Median Day 3 tremor intensity ratios for Groups 2 (lolitrem B intoxicated, no treatment) and 3 (lolitrem B intoxicated, acute KBr treatment.
** Significant difference between treated and untreated animals, p = <0.01.

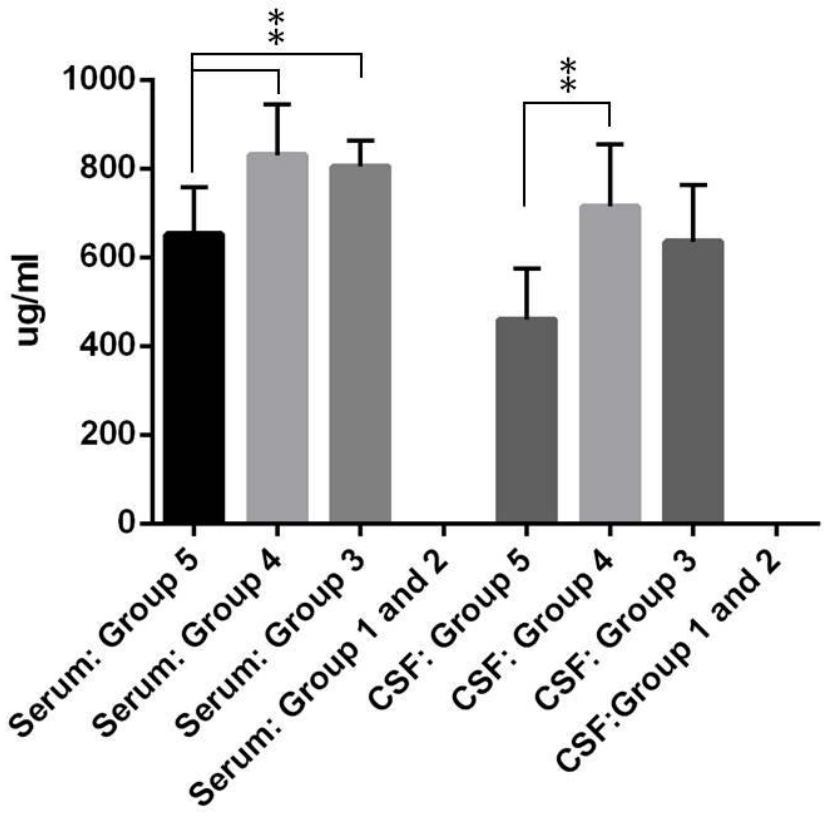

Figure 7. Bromide levels in serum and CSF are higher in lolitrem B intoxicated animals than in un-intoxicated controls. Serum: Group 5, n = 7; Group 4, n = 7; Group 3 n = 5. CSF: Group 5, n = 6; Group 5, n = 7; Group 3 n = 3.

3.4 Serum and CSF bromide levels are increased in the presence of lolitrem B toxin

To determine whether animals were maintaining prophylactic levels of bromide sufficient to be clinically effective after 22 days of administration, serum and CSF bromide concentrations were analysed on day of post mortem for all bromide treatment groups (Group 3: acute treatment 48 hours previously; 4: = bromide prophylactic treatment plus lolitrem B toxin for 22 days, and 5: bromide prophylactic treatment only for 22 days). Serum and CSF concentrations of bromide were found to be significantly higher in the bromide prophylactic treatment plus lolitrem B group (Group 4) than in the prophylactic treatment group 5 alone (see Figure 7) by a factor of 1.27:1 in serum and 1.57:1 in CSF. This finding was surprising and data suggest that animals intoxicated with lolitrem B in this study failed to excrete bromide with the same efficiency as their un-intoxicated counterparts thus maintaining higher levels of circulating bromide. The mean ratio of serum bromide: CSF bromide was also considered. This value was 0.74 for bromide prophylactic controls (Group 5); 0.91 for Bromide prophylactic treatment plus lolitrem B animals (Group 4) and 0.81 for acute bromide treatment plus lolitrem B animals (Group 3). This suggests that intoxicated animals treated with bromide might also maintain higher bromide levels in CSF compared to their un-intoxicated counterparts. This observation warrants further investigation as it suggests that very low levels of bromide might still deliver a prophylactic effect in cases of lolitrem B intoxication.

To investigate a possible mechanism for this difference, trace element content of the animal's feed was determined (DPI NWS Environmental Laboratory, Wollongbar, NSW). A very low level of chloride was found to be present in ryegrass seed compared to lucerne chaff (sodium: lucerne, 0.59%; ryegrass seed, 0.086%). Therefore, it is possible that this low level of chloride in the FOO to animals in the bromide prophylactic treatment Group 4 could have contributed to counter-current exchange of bromide ions leading to a higher intracellular concentration in these animals compared to their un-intoxicated counterparts who received a lucerne only diet.

3.5 Treatment with potassium bromide does not alter prevalence of neurological lesions observed in experimental cases of lolitrem B intoxication

Identification of early lesions associated with clinical presentation of experimental lolitrem B toxicity, and their correlation to lesions reported by us, and others, in naturally occurring field cases, was a key outcome of this study. The earliest lesions identified were restricted to the cerebellum and represent loss or dysfunction of neurons of the Purkinje layer and granule cell layer. The pyknotic nuclei observed in the granular layer of the cerebellum in this study likely represent evidence of granule neuron loss from this region, possibly via mechanisms of excitotoxic cell death, a finding that has not been reported previously (see Figure 1). The relative prevalence was not found to be significantly altered in any bromide treatment group, despite some differences in prevalence between Groups 2 and 3 particularly. These data suggest that treatment with potassium bromide does not mitigate underlying neuropathological cell damage associated with lolitrem B toxicity despite alleviating some of the clinical signs of toxicosis. Thus, the mode of action of the KBr treatment is not yet known.

Mild changes were noted in animals from Group 5 (bromide prophylactic treatment only animals) where one animal showed signs of a fine tremor and 4 animals were observed to show mild alterations in stance such as abnormal foot placement at rest. Anxiolysis was also noted in 5/9 animals in this group such that they were more placid and easy to handle than their Group 1 counterparts.

No overt neurological signs were observed in any animal within the control group (Group 1). In those animals presenting with neurological signs associated with lolitrem B toxicosis, no significant difference was observed between timing of onset of clinical signs between groups 2 and 3. However a significant difference was observed between the onset of clinical signs between groups 3 and 4 ($p = 0.026$) suggesting mild exacerbation of clinical presentation in these animals (see Table 3). Changes in body position (altered stance) was the major contributing factor to this result with altered stance being reported approximately two days earlier in this group than in their toxin-only counterparts (Groups 2 and 3, Table 2). A marginal increase was observed in the number of animals exhibiting truncal myoclonus compared to their toxin only counterparts, although this was not statistically significant. These data suggest that potassium bromide given at the prophylactic dose presented in this study is sufficient to induce mild mood changes in a significant proportion of animals although the penetration of this effect was variable and range of onset was wide (4 – 17 days; Table 2).

3.6 Alteration in renal function, but not hepatopathy, are observed in experimental cases of PRGT in sheep

Previously we had identified dehydration as playing a significant role in both clinical and preclinical cases of PRGT in the field(Combs, Rendell et al. 2014). In order to investigate if this was also the case in lolitrem B intoxication in an experimental setting, one in which animals had easy and convenient access to water *ad libitum*, we evaluated biochemical parameters associated with hydration status in our cohort. Evidence of dehydration was not observed in any parameters analysed either pre or post intoxication during this trial. A significant difference was observed between pre and post trial USG for group 2 only (toxin only) suggestive of a mild increase in urine concentration in this group (Group 2 pre-trial USG, mean = 1.0223, post-trial USG = 1.0381; p = 0.004; Table 4). This result is suggestive of reduced water intake or possibly increased faecal fluid losses with increasing intoxication in this group.

Table 4. Serum biochemistry analyte values pre- and post toxin and / or treatment for analytes indicative of hydration status.

Analyte	Group 1 - Control	Group 2 - Toxin only	Group 3 - Toxin + KBr Acute Treatment	Group 4 - Toxin + KBr Proph Treatment	Group 5 - KBr Proph Treatment	Reference range [a]
Packed Cell Volume (PCV)	35.10 (+/- 0.97) 35.57 (+/- 0.84)	34.42 (+/- 1.04) 37.71 (+/- 2.19)	32.57 (+/- 1.28) 34.28 (+/- 2.00)	32.71 (+/- 2.25) 34.28 (+/- 1.03)	34.57 (+/- 1.97) 33.42 (+/- 1.71)	0.29 – 0.40 l/l
Urine Specific Gravity (USG)	1.0248 (+/- 0.04) 1.0142 (+/- 0.03)	1.0223 (+/- 0.03) **1.0381 (+/- 0.04) ‡**	1.0263 (+/- 0.03) 1.0236 (+/- 0.04)	1.0212 (+/- 0.03) 1.0269 (+/- 0.05)	1.0324 (+/- 0.04) 1.0225 (+/- 0.06)	1.0218 – 1.0287[a]
Urea	8.41 (+/- 0.51) 7.63 (+/- 0.26)	8.68 (+/- 0.41) 6.04 (+/- 0.34) ‡	8.27 (+/- 0.38) 9.66 (+/- 4.25)	8.21 (+/- 0.34) 5.63 (+/- 0.38) ‡	8.56 (+/- 0.27) 6.96 (+/- 0.39)	5.4 – 11.4 mmol/L
Creatinine	69.8 (+/- 2.42) 74.7 (+/- 3.45)	68.5 (+/- 1.82) 84.6 (+/- 4.26)	66.1 (+/- 1.91) 79.2 (+/- 3.26)	74.6 (+/- 2.89) 82.4 (+/- 11.48)	73.0 (+/- 2.70) 77.0 (+/- 2.30)	44 – 72 mmol/L
Total serum protein	65.11 (+/- 0) 68.00 (+/- 0)	67.88 (+/-) 69.22 (+/-)	66.88 (+/- 1) 70.22 (+/- 0.)	68.44 (+/- 0.) 72.77 (+/- 0.)	68.44 (+/- 0.) 73.77 (+/- 1.)	59 – 80 g/L
Albumin	32.0 (+/- 0.50) 36.0 (+/- 0.69) ‡	32.0 (+/- 0.45) 35.0 (+/- 0.76) †	31.0 (+/- 1.40) 34.0 (+/- 0.92) ‡	32.0 (+/- 0.82) 33.0 (+/- 0.66)	31.0 (+/- 0.84) 33.0 (+/- 1.24)	29 – 41 g/L
GGT	46.0 (+/- 2.34) 49.3 (+/- 2.07)	56.11 (+/- 1.82) 65.22 (+/- 2.52) ‡	54.33 (+/- 3.85) 58.55 (+/- 3.73)	42.68 (+/- 4.81) 63.77 (+/- 2.82) †	52.11 (+/- 1.79) 56.55 (+/- 2.84)	47 – 95 U/L
GLDH	6.22 (+/- 1.46) 16.11 (+/- 6.11)	6.33 (+/- 1.00) **36.22** (+/- 12.76) †	9.77 (+/- 10.50) 21.66 (+/- 24.78)	4.66 (+/- 0.78) **30.77** (+/- 23.93)	12.22 (+/- 7.02) 7.66 (+/- 1.67)	0 – 23 U/L
Bilirubin	2.22 (+/- 0.44) 2.22 (+/- 0.44)	2.11 (+/- 0.33) 1.78 (+/- 0.66)	2.00 (+/- 0.33) 2.11 (+/- 0.33)	2.11 (+/- 0.44) 2.22 (+/- 0.50)	2.33 (+/- 0.50) 2.22 (+/- 0.44)	2 – 3 umol/L

[a] Reference range for experimental cohort derived from 95% confidence bootstrap interval taken from all samples for pre-trial animals.
† Significant difference between pre- and post trial samples, p = <0.05
‡ Significant difference between pre- and post trial samples, p = <0.01

Significant differences were observed between pre- and post trial urea values for Groups 2 and 3, (Group 2: p = 0.006; Group 3: p = 0.003) indicative of a mild hypouremia (Table 4). A significant difference was also observed between urea values recorded pre- and post trial for Groups 2 and 4 (Group 2: p = 0.002 in both cases) although no significance was noted for changes in urea values for Group 3 (see Table 4).

Post trial values for creatinine, a measure of glomerular filtration rate, and therefore normal kidney function, were observed marginally above normal range in all groups examined, with the highest values observed in Groups 2 and 4. The significance of this finding is unclear but might indicate subclinical renal dysfunction in correlation with the low urea values observed in these groups.

Albumin values also varied between the groups and some changes pre- to post treatment within groups were significant (Table 3), however, none represented changes outside normal ranges. Values for total protein, PCV (see Table 4), sodium and chloride (data not shown), pre- and post trial, were not significantly different and did not represent changes outside normal ranges.

Together this data suggest a trend towards changes indicative of renal dysfunction in animals suffering from lolitrem B intoxication without reaching level indicative of overt clinical significance. These data suggest that mild dehydration was evident in lolitrem B intoxicated animals in the absence of treatment with potassium bromide but that severe dehydration was not a clinical feature of experimental PRGT, likely due to the animal's convenient and available access to water despite increasing intoxication.

Analysis of liver function in our cohorts did not reveal any significant hepatopathy, however, a mild, statistically significant, increase in the liver enzyme gamma glutyl-transpeptidase (GGT) were observed in our toxin only animals (Group 2) and toxin plus bromide prophylactic treatment animals (Group 4) although these values did not fall outside the normal reference range for our study (see Table 3). Mild increases were also noted in pre- to post trial glutamate dehydrogenase (GLDH) in Groups 2 and 4, both representing increases to marginally above normal reference range, however, only the change in group 2 reached significance (p = 0.45). No significant difference was observed in bilirubin pre- or post trial in any group examined (see Table 4). Together these data suggest that lolitrem B does not have hepatotoxic properties, nor is hepatopathy a significant contributory feature of experimental lolitrem B intoxication.

4 Discussion

The findings in this study represent a significant breakthrough in available treatment options for the neurological deficits associated with perennial ryegrass toxicosis. The dramatic improvements in gait observed in this study and the ability to ablate tremor without sedation on delivery of an acute oral dose of potassium bromide are unique characteristics of this therapy. Its ease of administration and long half life in the animal make it an ideal therapeutic intervention for this plant toxicity.

To date, no successful therapeutic has been brought to market for PRGT. Despite some indication of efficacy with mycotoxin binding agents (Reed, Cummins et al. 2011), they have limited efficacy in the field, particularly in severe outbreaks, and cannot be used as a therapeutic modality for severely affected animals. Pasture renovation with novel endophyte-infested varieties of perennial ryegrass is a viable option for disease mitigation however these varieties currently suffer from a range of issues including poor persistence in the Australian environment, the production of other toxic agents and as yet unclear long term economic advantage over wild type endophyte-infested pastures. Given this complex interplay between nature, pasture, environment and the disease, and the difficulty in

manipulating all these factors to produce a 'safe' pasture, an effective therapeutic, such as bromide, provides an important alternative approach to disease management for Australian producers.

A number of clinical observations made during this study may a have relevance to field cases of PRGT. This study identifies the first controlled observations of neurohistopathological changes associated with the pathogenesis of ryegrass 'staggers' in sheep. Cerebellar changes, including the presence of spheroids in the granular cell layer, pyknotic granular cell nuclei and Purkinje cell vacuolation, were observed in all lolitrem B intoxicated animals but were absent in control animals. This confirms these changes as the earliest lesions observable in clinical cases of rye Grass Staggers. These finding should aid to clarify what has previously been a controversial diagnostic area by confirming presence of spheroids and a reduction in cell density in the granular layer of the cerebellum as two key diagnostic features of lolitrem B toxicity. Myositis, which has been reported in field cases of PRGT by us (Combs, Rendell et al. 2014) and others (Reed, Vaughan et al. 2010), was not observed in our experimental cases, either on serum biochemistry or histopathology. This suggest that the aetiology of these disease manifestations is likely to be due to repeated falling rather than being a direct affect of the toxicoses, or by other unidentified toxic metabolites produced by natural endophytes.

Response to bromide prophylaxis showed positive clinical effects with animals showing less severe gait abnormalities than their intoxicated counterparts. The time to necropsy for this group was decided as 22 days on the basis of our previous PK study which indicated that serum concentrations of bromide would be depleted below therapeutic levels by this date. This proved, on analysis of serum and CSF in our prophylactic treatment group not to be the case. There are a number of potential explanations for this finding which warrant further investigation. Serum bromide levels were found to be significantly higher in animals administered a prophylactic dose and exposed to intoxicating feed compared to those administered bromide alone. As a simple ion, bromide is taken across the cell membrane through the same calcium-dependent ion channels which generate chloride gradients (Geck and Heinz 1986, Rocha-Gonzalez, Mao et al. 2008), their role to generate a high level of intracellular chloride and, in the case of neurons, hyperpolarising the cell (Alvarez-Leefmans, Gamino et al. 1988). This action of chloride is important as it modulates activity of GABA-ergic inhibitory neurons in the central nervous system, such as those of the Purkinje layer of the cerebellum. Bromide ions have been shown to be selectively transported with chloride ions in this active inward transport system (Simchowitz 1988) suggesting that, in the presence of low Cl^- levels, bromide might be actively transported in an increasing intracellular gradient across the cell membrane. Significant variation in Cl levels between the two feeds (lucerne and ryegrass seed) were identified as a possible source of this difference. Furthermore, a direct interference with bromide excretion by renal calcium-dependent BK channel blockade (such as is the case in lolitrem B intoxication), channels which are known to play a role in ion homeostasis(Rieg, Vallon et al. 2007), cannot be excluded as a potential mechanism for the systemic bromide retention observed in our intoxicated bromide treated animals. Together these data suggest that bromide has potential to be an effective preventative with efficacy at doses lower than those used for prophylaxis in this trial. This treatment should be replicated with a lower dose delivered over a longer period of time to confirm this hypothesis.

This study is the first to report serum concentrations of bromide used therapeutically. Upper and lower limits of efficacy of potassium bromide in sheep have not yet been defined. In addition to experiencing systemic retention of bromide in the presence of lolitrem B toxicosis, it is possible that

sheep are more sensitive to treatment with oral potassium bromide than monogastric species, from which the dose levels in this study were extrapolated. At a therapeutic dose of 500mg/kg we observed clear signs of sedation, albeit mild, in our pre-trial animals. This was considered undesirable from a production perspective, and so a lower dose was used for therapy with a staggered delivery used for our prophylactic cohorts.

A heightened sensitivity to a neuroactive compound is not an unexpected suggestion. Sheep (and other ruminants) are approximately ten times more sensitive to the sedating effects of xylazine than monogastrics, which is reflected in the much lower doses of this drug used in sheep(Lin 2014). The sedation observed in the prophylactic group where, on average, serum bromide levels were found to be higher than the bromide only controls, supports a dose response relationship. Further trials need to be undertaken to examine effects of potassium bromide at these dose levels in the absence of lolitrem B toxicosis as well as refining the dose for intoxicated animals.

5 Conclusions

We have established a reliable and reproducible experimental model to study lolitrem B toxicosis in sheep which mimics closely those neurological clinical signs observed in field cases of PRGT. Data from this study suggest bromide to be an effective therapeutic treatment for Rye Grass Staggers in sheep. Due to its efficacy and the ability of the drug to be administered orally as well as an anticipated low cost per treatment to the farmer this treatment represents a practical solution for the neurological deficits associated with PRGT. APVMA registration and the development of practical on-farm treatment formulations and recommendations are the next steps towards this drug becoming available to farmers to mitigate clinical cases of Rye Grass Staggers on farm and further studies will likely determine the most effective dose for prophylaxis in addition to an acute treatment

6 Appendices

Appendix 1:

Corrected reference intervals for haematology and biochemistry in mixed breed lambs, as determined by normal or non-parametric data distributions. See Appendices for tables outlining the normal and parametric datasets. [b] Where the lower bound of the reference interval was x < 0, the value was corrected to have 0 as a biologically feasible lower limit.

		Chosen Reference Interval	
Analyte	**Units**	**Lower**	**Upper**
PCV	l/l	0.29	0.40
Total protein	g/L	59	80
Albumin	g/L	29	41
Globulins	g/L	21	47
A:G ratio		0.6	1.7
Creatinine	umol/L	44	72
Urea	mmol/L	5.4	11.4
CK	U/L	0[b]	1194
AST	U/L	59	130
GLDH	U/L	0[b]	23
Bilirubin	umol/L	2	3
GGT	U/L	47	95
Potassium	mmol/L	4.3	5.5
Sodium	mmol/L	137	148
Na:K ratio		24.9	33.9
Anion gap	mmol/L	12	23
Bicarbonate	mmol/L	19	32
Calcium	mmol/L	2.81	3.42
Phosphate	mmol/L	1.02	2.67
Ca:PO4 ratio		0.86	2.92
Chloride	mmol/L	101	112

7 Bibliography

Alvarez-Leefmans, F. J., S. M. Gamino, F. Giraldez and I. Nogueron (1988). "Intracellular chloride regulation in amphibian dorsal root ganglion neurones studied with ion-selective microelectrodes." J Physiol **406**: 225-246.

Cheeke, P. R. (1995). "Endogenous toxins amd mycotoxins in forage grasses and their effects on livestock. ." Journal of animal science **73**(3): 909-918.

Combs, M., D. Rendell, K. Reed, W. Mace and J. Quinn (2014). "Evidence of dehydration and electrolyte disturbances in cases of perennial ryegrass toxicosis in Australian sheep." Aust Vet J **92**(4): 107-113.

Cunningham, I. J. a. H., W.J. (1959). "Ryegrass staggers." New Zealand Veterinary Journal **7**: 1-7.

Dixon, W. (1953). "processing data for outliers." Biometrics: 74-89.

Finnie, J. W., P. A. Windsor and A. E. Kessell (2011). "Neurological diseases of ruminant livestock in Australia. I: general neurological examination, necropsy procedures and neurological manifestations of systemic disease, trauma and neoplasia." Australian veterinary journal **89**(7): 243-246.

Gallagher, R. T., A. G. Campbell, A. D. Hawkes, P. T. Holland, D. A. McGaveston, E. A. Pansier and I. C. Harvey (1982). "Ryegrass staggers - the presence of lolitrem neurotoxins in perennial ryegrass seed." New Zealand Veterinary Journal **30**(11): 183-184.

Gallagher, R. T., A. D. Hawkes, P. S. Steyn and R. Vleggaar (1984). "Tremorgenic Neurotoxins from Perennial Ryegrass Causing Ryegrass Staggers Disorder of Livestock - Structure Elucidation of Lolitrem-B." Journal of the Chemical Society-Chemical Communications(9): 614-616.

Gallagher, R. T., E. P. White and P. H. Mortimer (1981). "Ryegrass Staggers - Isolation of Potent Neurotoxins Lolitrem a and Lolitrem-B from Staggers-Producing Pastures." New Zealand veterinary journal **29**(10): 189-190.

Geck, P. and E. Heinz (1986). "The Na-K-2Cl cotransport system." J Membr Biol **91**(2): 97-105.

Lin, H. C., Walz, P., Ed. (2014). Farm Animal Anesthesia: Cattle, Small Ruminants, Camelids, and Pigs. Oxford, United Kingdom, John Wiley and Sons.

Mayhew, J. I. G. (2009). Large Animal Neurology. Oxford, United Kingdom, John Wiley and Sons Ltd.

Parton K, B. A., Chambers JP. (2006). Veterinary Clinical Toxicology., Massey University.

Podell, M. and W. R. Fenner (1993). "Bromide Therapy in Refractory Canine Idiopathic Epilepsy." Journal of Veterinary Internal Medicine **7**(5): 318-327.

Reed, A. H., Henry, R.J., Mason, W.B. (1971). "Influence of statistical method used on the resulting influence of normal range. ." Clinical Chemistry **17**: 275-284.

Reed, K. F. M., L. J. Cummins, D. D. Moore and A. J. Clark (2011). "Performance of Coopworth ewe lambs exposed to low levels of ryegrass endophyte (Neotyphodium lolii) alkaloids and allowed access to a mycotoxin deactivator." Animal Production Science **51**(3): 225-232.

Reed, K. F. M., Z. N. Nie, L. V. Walker, W. J. Mace and S. G. Clark (2011). "Weather and pasture characteristics associated with outbreaks of perennial ryegrass toxicosis in southern Australia." Animal Production Science **51**(8): 738-752.

Reed, K. F. M., J. L. Vaughan, L. J. Cummins and D. D. Moore (2010). "Impact of mycotoxins and of a mycotoxin deactivator on alpacas grazing perennial ryegrass infected with wild endophyte (Neotyphodium spp.)." Animal Production Science **50**(9): 902-908.

Rieg, T., V. Vallon, M. Sausbier, U. Sausbier, B. Kaissling, P. Ruth and H. Osswald (2007). "The role of the BK channel in potassium homeostasis and flow-induced renal potassium excretion." Kidney international **72**(5): 566-573.

Rocha-Gonzalez, H. I., S. Mao and F. J. Alvarez-Leefmans (2008). "Na+,K+,2Cl- cotransport and intracellular chloride regulation in rat primary sensory neurons: thermodynamic and kinetic aspects." J Neurophysiol **100**(1): 169-184.

Sackett D, F. J. (2006). 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. . North Sydney, Australia.

Simchowitz, L. (1988). "Interactions of bromide, iodide, and fluoride with the pathways of chloride transport and diffusion in human neutrophils." J Gen Physiol **91**(6): 835-860.

Tietz, N. W. (1976). Fundamentals of clinical chemistry.