CLINICAL AND PATHOLOGICAL CHARACTERISATION OF PERENNIAL RYEGRASS TOXICOSIS AND INVESTIGATION OF BROMIDE AS A THERAPEUTIC INTERVENTION

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This thesis is presented for the degree of Doctor of Philosophy at Charles Sturt University August 2019
Cover image: Vestibulocerebellum from an animal suffering from perennial ryegrass toxicosis
# TABLE OF CONTENTS

**List of Figures** ............................................................................................................. x

**List of Tables** .................................................................................................................. xiv

**Certificate of Authorship** ................................................................................................ xx

**Acknowledgements** ....................................................................................................... xxi

**List of Publications and Conference Proceedings** ....................................................... xxvii

**List of Abbreviations** .................................................................................................... xxx

**Abstract** .......................................................................................................................... xxxii

**Chapter 1 Introduction** .................................................................................................. 1

**Chapter 2 Literature Review** ......................................................................................... 8

2.1 Perennial Ryegrass Toxicosis, Lolitrem B and the Investigation of Therapeutic Agents, including Bromide...................................................................................................................... 8

2.2 Estimations of Economic Losses and Farming Impacts.................................................. 10

2.3 Understanding the clinical syndrome: Perennial Ryegrass Toxicosis............................... 11

2.3.1 Use of ryegrass cultivars and reports of PRGT from the 19th century......................... 11

2.3.2 Clinical Observations 1880-1959 ........................................................................... 11

2.4 Histopathological Lesions Associated with PRGT (1959-2008) .................................... 12

2.5 Understanding the Plant-Fungus-Toxin Interaction .......................................................... 13

2.5.1 Identification of association with *Epichloë festucae var. lolii* infested perennial rye grass (*Lolium perenne* L.) and PRGT................................................................. 13

2.5.2 Identification of lolitrem B toxin as primary toxic agent ............................................... 15

2.6 Understanding Toxin-Animal Interactions ...................................................................... 16

2.6.1 Mechanism of action of lolitrem B toxin .................................................................... 16

2.6.2 Subclinical production losses and the role of other fungal alkaloids in PRGT. 18

2.7 Laboratory Models of PRGT ........................................................................................ 20

2.7.1 Sheep Models of Perennial Ryegrass Toxicosis ......................................................... 21

2.7.2 Cattle Models of PRGT ............................................................................................ 25

2.7.3 Equine Model of PRGT ............................................................................................ 27

2.7.4 Camel Model of PRGT .............................................................................................. 28

2.7.5 Rodent Models of PRGT ........................................................................................... 30

2.7.6 Summary of PRGT Animal Models .......................................................................... 37

2.8 Potential Therapeutic Agents for Treatment of PRGT .................................................... 40

2.8.1 Bromide .................................................................................................................. 40
2.8.2 Bromide Mechanism of Action ................................................................. 42
2.8.3 Bromide Pharmacokinetics ................................................................. 43
2.8.4 Analysis of Serum Bromide ................................................................. 45
2.8.5 Other Potential Therapeutic Agents .................................................... 45
2.8.6 Other Management Strategies ............................................................. 47
2.9 Hypotheses and Research Objectives .................................................... 48

Chapter 3 Clinical Presentation of Perennial Ryegrass Toxicosis Outbreak in Australia ................................................................. 50

3.1 Introduction ............................................................................................. 52
3.2 Case Series ............................................................................................. 53
  3.2.1 History ............................................................................................. 53
  3.2.2 Inclusion Criteria ............................................................................. 54
  3.2.3 Animals ........................................................................................... 54
  3.2.4 Sample collection ............................................................................ 57
  3.2.5 Neurological Signs ......................................................................... 58
  3.2.6 Pathological Analysis ..................................................................... 59
  3.2.7 Toxin Quantification in Plant and Animal Samples ......................... 61
3.3 Discussion ............................................................................................... 62
  3.3.1 Effects of Dehydration in PRGT Cases ............................................ 64
  3.3.2 Acknowledgements ......................................................................... 68

Chapter 4 Characterisation of Neurological Dysfunction induced by the BK Channel Antagonists Paxilline and Lolitrem B using a Mouse Model ........................................... 69

4.1 Introduction ............................................................................................. 73
4.2 Materials and Methods ........................................................................ 74
  4.2.1 Animals ........................................................................................... 74
  4.2.2 Toxins ............................................................................................ 75
  4.2.3 Experimental Design ..................................................................... 75
  4.2.4 Tremor Analysis ............................................................................. 76
  4.2.5 Coordination and Voluntary Motor Activity .................................. 77
  4.2.6 Spatial Learning and Memory ......................................................... 79
  4.2.7 Novel Object Recognition (NOR) Test .......................................... 80
  4.2.8 Statistical Analysis ......................................................................... 80
4.3 Results .................................................................................................... 80
  4.3.1 Piezoelectric Tremor Analysis Identifies Length of Tremorgenic Action of Lolitrem B Toxin in Mice after a Single Exposure .................................................. 80
4.3.2 Tremorgenic Toxins Lolitrem B and Paxilline Inhibit Normal Voluntary Movement in Intoxicated Mice after a Single Exposure ............................................................ 83

4.3.3 Lolitrem B and Paxilline Impair Voluntary Movement but Not Motor Coordination .................................................................................................................. 84

4.3.4 Paxilline and Lolitrem B Impedes Motor Performance but Not Positive Bias or Exploratory Behaviour in Intoxicated Mice ......................................................... 86

4.3.5 Lolitrem B Intoxication Affects Spatial Orientation but Not Spatial Learning and Memory ................................................................................................................... 88

4.4 Discussion .................................................................................................................... 90

4.4.1 Sensitive Quantification and Characterisation of Tremor can be Achieved using Piezoelectric Pressure Sensor Analysis ......................................................... 91

4.4.2 Lolitrem B Intoxication Impairs Spatial Orientation but Not Visual, Contextual or Spatial Learning and Memory ......................................................... 92

4.4.3 Both lolitrem B and Paxilline Inhibits Voluntary Movement Acutely in Mice after a Single Toxic Exposure Despite Continued Tremor ........................................ 92

4.4.4 Lolitrem B and Paxilline Intoxicated Animals Show Inhibition of Locomotion Without Classical Ill-coordination ........................................ 94

4.5 Conclusion .................................................................................................................. 94

4.6 Acknowledgements .................................................................................................... 95

Chapter 5 Bromide Reduces Tremor and Decreases Perception of Stress after a Single Exposure to Lolitrem B in Mice ................................................................. 96

5.1 Introduction .................................................................................................................. 97

5.2 Materials and Methods .............................................................................................. 99

5.2.1 Animals .................................................................................................................. 99

5.2.2 Treatments ........................................................................................................... 99

5.2.3 Protocols .............................................................................................................. 99

5.2.4 Toxin Formulation .............................................................................................. 100

5.2.5 Tremor Analysis .................................................................................................. 100

5.2.6 Behavioural Testing ........................................................................................... 100

5.2.7 Coordination and Voluntary Motor Activity Testing .......................................... 101

5.2.8 c-Fos Immunoreactivity ..................................................................................... 101

5.2.9 Statistical analysis .............................................................................................. 102

5.3 Results ....................................................................................................................... 103

5.3.1 Oral Bromide Therapy Reduces Lolitrem B Induced Tremor in Rodents .... 103

5.3.2 Bromide Therapy Reduces Length of Freezing Episodes and Increases Exploratory Behaviour in Lolitrem B Intoxicated Mice ........................................ 105

5.3.3 Bromide Treatment Causes Behavioural Disinhibition but does Not Appear to Improve Coordination in Lolitrem B Intoxicated Mice .................................... 108
5.3.4 Bromide pre-treatment reduces perception of stress, but not neuro-endocrine pathway responses to lolitrem B intoxication.......................................................... 109
5.4 Discussion ................................................................................................................. 113
5.4.1 Bromide Reduces Lolitrem B Induced Tremor and Improves Mobility of Lolitrem B Intoxicated Mice. .......................................................... 113
5.4.2 Bromide Reduces Perception of Stress but Not Physiological Stress in Lolitrem B Intoxicated Mice .......................................................... 114
5.4.3 Bromide Reduces Lolitrem B Inhibition of Movement but Does Not Improve Conscious Proprioception .......................................................... 114
5.4.4 Implications for Bromide Therapy of Tremor Syndromes of Cerebellar Origin 115
5.4.5 Conclusion: Bromide is a Potential Therapeutic for PRGT .......................... 116

Chapter 6 Development of a Model for Investigation of Perennial Ryegrass Toxicosis in Sheep 118

6.1 Introduction ................................................................................................................. 121
6.2 Materials and Methods .............................................................................................. 123
6.2.1 Animals .................................................................................................................. 123
6.2.2 Treatments ............................................................................................................. 124
6.2.3 Testing Phase .......................................................................................................... 125
6.2.4 Clinical signs including observation of gait .......................................................... 125
6.2.5 Clinical Chemistry Procedures .............................................................................. 126
6.2.6 Measurement of Water Intake and Live Weight Gain ........................................... 127
6.2.7 Faecal Cortisol Analysis ...................................................................................... 127
6.2.8 Electromyography ................................................................................................. 127
6.2.9 Mechatronic Sensory Nociceptive Threshold Testing ........................................... 128
6.2.10 Pathological Examination ................................................................................... 128
6.2.11 Statistical Analysis .............................................................................................. 129
6.3 Results ........................................................................................................................ 130
6.3.1 Neurological Abnormalities, Gait Disorder and Clinical Changes in Intoxicated Sheep 130
6.3.2 Clinical Pathology ................................................................................................. 135
6.3.3 Water Intake and Weight ...................................................................................... 137
6.3.4 Faecal Cortisol Analysis ...................................................................................... 137
6.3.5 Surface Electromyography ................................................................................... 138
6.3.6 Mechatronic Sensory Nociceptive Threshold Testing ........................................... 139
6.3.7 Histopathological Changes ................................................................................... 140
6.4 Discussion ................................................................................................................... 141

vi
Chapter 7 Treatment with Potassium Bromide Mitigates Ataxia and Reduces Tremor in Lambs with Perennial Ryegrass Toxicosis

7.1 Introduction
7.2 Materials and methods
7.2.1 Animals
7.2.2 Treatments
7.2.3 Feeding Phase
7.2.4 Testing Phase
7.2.5 Treatment with KBr
7.2.6 Electromyography
7.2.7 Measurement of Bromide in Serum and Cerebrospinal Fluid
7.2.8 Concentrations of Cortisol Metabolites in Faeces
7.2.9 Histopathology
7.2.10 Statistical Analyses
7.3 Results
7.3.1 Neurological Observations and Gait Assessment
7.3.2 Electromyography
7.3.3 Concentrations of Bromide in Serum and CSF
7.3.4 Concentrations of Cortisol Metabolites in Faeces
7.3.5 Histopathology
7.4 Discussion
7.5 Acknowledgements

Chapter 8 Discussion
8.1 Scope of Study
8.2 Advancements in the Clinical/Laboratory Assessment of PRGT
8.3 Advancements in Understanding of Aetiopathogenesis of PRGT
8.4 Advancements in Therapeutic Options for PRGT
8.5 Comparative Aspects of Research
8.6 Overall Outcome Summary

Chapter 9 References

Chapter 10 Appendices
10.1 Appendix 1: Data Relating to Chapter 3 Clinical Presentation of perennial Ryegrass Toxicosis Outbreak in Australia
10.1.1 Farm Locations Visited During Chapter 3 Study
10.4.5 Feeding Phase Day 1 (entry) and Testing Phase Day 2 (exit) Urinalysis and PCV  218

10.5 Appendix 5: Patent for treatment of toxicosis in sheep.................................219

10.6 Appendix 6: Patent for Treatment of Stress in Grazing Animals......................248

10.7 Appendix 7: Conference proceeding and poster presentations: .......................268

10.7.1 2011 Poster Submission: Sheep CRC/ MLA Postgraduate Conference.....268

10.7.2 Poster Submission: Australian Neuroscience Society 2013 .......................269

10.7.3 Australian Neuroscience Society 2016 ...................................................270

10.7.4 Poster Presentation: Australian Sheep Vets Conference 2016 .................271


10.7.7 Conference Proceedings: Neuroscience 2013, San Diego .......................288

10.7.8 Conference Proceedings: Perennial Ryegrass Toxicosis; Investigating the Complex Biochemistry of Shaky Sheep. RACI, NPCG, 2019 .....................290

10.7.9 Poster Presentation: Neuroscience 2013, San Diego ..............................292

10.7.10 Bromide Pharmacokinetics in Sheep: Published AVJ 2015 .................293
LIST OF FIGURES

Figure 2-1: Histological section of the cerebellum from a sheep affected by perennial ryegrass toxicosis. 13

Figure 2-2: A scanning electron microscope image of *Epichloë festucae var. lolii* mycelium growing on the surface of a perennial ryegrass leaf. 14

Figure 2-3: Lolitrem B molecule structure and biosynthetic pathway 16

Figure 3-1: Histological section of the cerebellum from a sheep affected by perennial ryegrass toxicosis. 60

Figure 3-2: Lolitrem B and ergovaline concentrations from replicate samples of perennial ryegrass from affected paddocks on seven farms where grazing livestock exhibited clinical signs of perennial ryegrass toxicosis during autumn 2011. 62

Figure 4-1: Time line of sequence and timing of tremor and behavioural tests over 4 days 76

Figure 4-2: Fast Fourier Transformed (FFT) tremor frequencies in lolitrem B and untreated control mice. 78

Figure 4-3 Comparison of Tremor Motion Power Ratio and Peak Tremor Frequency in lolitrem B, paxilline, vehicle control and no treatment control mice until 72 hours post treatment. 82

Figure 4-4 Measurement of motor coordination in loitrem B and paxilline intoxicated mice using parallel rod apparatus 85

Figure 4-5: Analysis of optimistic exploratory behavior and movement using the novel object recognition test. 87

Figure 4-6: Barnes maze occupancy plots from four lolitrem B intoxicated mice at 3 and 27 hours post intoxication. 88

Figure 4-7: Barnes maze latency to escape hole entry for mice 3, 27 and 51 hours after single exposure to lolitrem B or paxilline and compared to vehicle or untreated controls. 89
Figure 4-8: Barnes maze inactivity at 3 hours post injection of toxin.

Figure 5-1: FFT analysis of tremor, at 60min post lolitrem B injection and no treatment or bromide pre-treated.

Figure 5-2: Tremor: Motion Power Ratio (TMPR) following lolitrem B injection.

Figure 5-3: Comparison of distance travelled in novel arena test.

Figure 5-4: Freezing behaviour within the novel arena test

Figure 5-5: Parallel Rod Touches

Figure 5-6: Maximum Speed within parallel rod test.

Figure 5-7: Photomicrographs

Figure 5-8: Neuronal cell body counts for c-Fos immunoreactivity: in the paraventricular nuclei and central amygdala.

Figure 6-1: Mean (a) rectal temperature, (b) respiration rate, and (c) heart rate, in lambs fed perennial ryegrass seed containing lolitrem B and ergotamine, and control lambs.

Figure 6-2: Mean concentrations of cortisol metabolites in faeces collected at necropsy from lambs fed perennial ryegrass seed containing lolitrem B and ergotamine, and control lambs.

Figure 6-3: Geometric mean root mean square voltage of surface electromyograms recorded from the triceps muscle of lambs fed perennial ryegrass seed containing lolitrem B and ergotamine.

Figure 6-4: Mean mechanosensory nociceptive threshold recorded in lambs fed perennial ryegrass seed containing lolitrem B and ergotamine.

Figure 6-5: Photomicrograph of a section of the vestibulocerebellum of a lamb fed perennial ryegrass seed containing lolitrem B and ergotamine.
Figure 7-1. Geometric mean root mean square voltages of surface electromyograms recorded from the triceps muscle of lambs 161

Figure 7-2: Mean concentrations of cortisol metabolites in faeces collected at necropsy from lambs 163

Figure 10-1: Locations of farms visited during 2011 outbreak of PRGT in South-West Victoria 196

Figure 10-2: A paddock from which animals were removed following demonstrating signs of PRGT. 197

Figure 10-3: Identification of Ryegrass varieties in paddock. 197

Figure 10-4: A merino lamb that fell into lateral recumbency during moving stock. 200

Figure 10-5: Sheep A1: An animal that has been in prolong recumbency 201

Figure 10-6: Sheep D10. This animal has fallen near a fence and in its struggles to stand has become trapped under a fence. 201

Figure 10-7: Several other animals on the same property remained in sternal recumbency. 202

Figure 10-8: Sheep D6 and D8: Histological section of the cerebellum. 203

Figure 10-9: A composite Fast Fourier Transform graph of power-frequency spectrum at all time-points within the tremor characterisation study. 204

Figure 10-10: Tremor-Motion Power Ratio across the 72 hour testing period in lotitrem B intoxicated mice. 205

Figure 10-11: Paxilline Tremor-Motion Power Ratio across 5 hours 205

Figure 10-12: Effect of paxilline and lolitrem B on distance travelled by parallel rod test. 206

Figure 10-13: Composite FFT from piezoelectric pressure sensor in paxilline intoxicated mice on each day of behavioural trial 207
Figure 10-14: Composite FFT from piezoelectric pressure sensor in mice receiving a 90% DMSO injected mice on each day of behavioural trial. 208

Figure 10-15: Video Capture from novel object recognition test. 209

Figure 10-16: Examples of head tracking heat map plots a series of hours post lolitrem B or no treatment control. 210

Figure 10-17: ANY-maze™ Parallel Rod Apparatus. 211

Figure 10-18: Camera view of the Barnes Maze as tracked by ANY-maze™ software. 212

Figure 10-19: Comparison of voluntary movement on parallel rod in lolitrem B intoxicated animals, 1 hour post treatment with a potential therapeutic agents. 213

Figure 10-20: Sheep showing signs clinical consistant with PRGT and meeting criteria for inclusion into Testing Phase of trial. 215
LIST OF TABLES

Table 2-1: The system of scoring generated by Keogh (1973) used to assess severity of ryegrass staggers clinical signs in sheep, known as ‘The Keogh Scale’. 22

Table 2-2: Blythe scoring system developed to assess severity of ryegrass staggers in cattle (Blythe 2007). 26

Table 2-3: Visual rating scale for tremor assessment in rodents (Gallagher and Hawkes 1986) 32

Table 3-1: Clinical findings for 13 sheep affected by perennial rye grass toxicosis and sampled during autumn 2011 in south-western Victoria, Australia 56

Table 6-1: Progression of behavioural and neurological signs in PRGT model 132

Table 6-2: mean (± sem) serum biochemistry analyte, packed cell volume (pcv) and urine specific gravity (usg) values in control and treatment group animals 136

Table 7-1: Description of treatment groups for lambs included in a study to assess the use of potassium bromide in sheep being fed endophyte infested ryegrass seed containing lolitrem B. 152

Table 7-2: Clinical observations made during gait assessment of lambs included in a study to assess the use of potassium bromide in sheep being fed endophyte infested ryegrass seed containing lolitrem B. 154

Table 7-3: Mean composite gait scores and proportion of animals falling during gait assessment in the Testing phase of a trial to assess the effect of potassium bromide in lambs being fed endophyte infested ryegrass seed containing lolitrem B. 160

Table 7-4: Mean concentrations of bromide in serum and cerebrospinal fluid measured following euthanasia on Day 2 of the Testing phase of a trial to assess the effect of potassium bromide in lambs being fed endophyte infested ryegrass seed containing lolitrem B. 162

Table 10-1: HPLC Analysis of pasture samples taken during the 2011 PRGT outbreak in SW Victoria. 198

Table 10-2: Basic feed analysis on ryegrass seed. 214

Table 10-3: Feeding phase day 1 serum biochemistry results. 216

Table 10-4: Testing phase day 2 serum biochemistry. 217

Table 10-5: Feeding phase day 1 and testing phase day 2 urinalysis and PCV 218
Certificate of Authorship

I hereby declare that this submission is my own work and to the best of my knowledge and belief, understand that it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged.

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Name           Martin Combs
Signature       
Date           8 August 2019
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CSU requires a declaration of the following as per Schedule 3 of the Thesis Requirements

a. title, authorship and publication outlet of each paper;

b. the current status of each paper;

c. the extent of the contribution of the candidate to the research and the authorship of each paper.

As per Schedule Three of thesis requirements, I declare acknowledgement of the following assistance with the research papers presented as part of this thesis:

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The contribution of the candidate to this chapter was as follows:

Clinical interview of clients, physical examination and neurological examinations of animals, collection of clinicopathological specimens, conducting necropsies and collecting histopathological samples, interpretation of clinical pathology data and faecal and pasture alkaloid concentrations in light of physical examination findings and clinical history, examination of neurological histopathology with the assistance of S Raidal and A Kessel, examination of pastures.

The text was authored by the candidate with review of drafts by J Quinn.

Other contributors include: CSU Veterinary Diagnostic Laboratory, C Farish, S Raidal and A Kessel provided serum biochemistry analysis and processing of histopathological samples. Southern Scientific Services Ltd, Hamilton, provided pasture toxin analysis. W Mace, AgResearch Limited, Grasslands Research Centre, Palmerston North, New Zealand provided additional pasture analysis. K Reed, provided technical advice on pasture sampling and assisted access to affected farms. D Rendell provided local veterinary knowledge regarding outbreaks of PRGT, assisted with clinical examinations.
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Other contributors include: G Rogers assisted with husbandry and neurological testing of mice including collection of preliminary data. J Quinn assisted with neurological testing and development of testing/animal housing facilities. A Hamlin advised on neurological testing protocols, provided laboratory equipment and assisted with statistical analysis. S Finch provided lolitrem B and paxilline toxins.

CHAPTER 5

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Signed:

Candidate: ___________________________ Date: 8 August 2019

Dr Martin Combs

Senior Author: ___________________________ Date: 5 August 2019

Associate Prof. Jane Quinn
List of Publications and Conference Proceedings

The following list outlines the collection of papers, patent requirements, conference proceedings and posters resulting from the research of the PhD project.

Journal Articles

**Combs MDA**, Rendell D, Reed KFM, Mace WJ, Quinn JC. Evidence of dehydration and electrolyte disturbances in cases of perennial ryegrass toxicosis in Australian sheep. *Australian Veterinary Journal* 92, 107-13, 2014

**Combs MD**, Hamlin A, Quinn JC. A single exposure to the tremorgenic mycotoxin lolitrem B inhibits voluntary motor activity and spatial orientation but not spatial learning or memory in mice. *Toxicon*, 2019
doi:https://doi.org/10.1016/j.toxicon.2019.06.228


Quast T, **Combs M**, Edwards S. Pharmacokinetics of bromide in adult sheep following oral and intravenous administration. *Australian Veterinary Journal* 93, 20-5, 2015

Patents

Patent no. US15/038,312; AU2014353885A, **Combs M**, Quinn J, Edwards S. Prevention and treatment of toxicosis. (Charles Sturt University, James Hagan Court, Boorooma Street, Wagga Wagga New South Wales 2678, Australia) 2015
Provisional Patent no. 15/038,261; AU2013904516 Combs M, Quinn J, Edwards S. Stress management in livestock. (Charles Sturt University, James Hagan Court, Boorooma Street, Wagga Wagga, New South Wales, 2678, Australia) 2015

Conference proceedings and poster presentations


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List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>avidin-biotin complex</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BK</td>
<td>calcium activated potassium</td>
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<tr>
<td>BUN</td>
<td>blood urea nitrogen</td>
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<td>CBC</td>
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<td>CRF</td>
<td>corticotrophin releasing factor</td>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<tr>
<td>EMG</td>
<td>electromyography</td>
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<tr>
<td>FCM</td>
<td>faecal cortisol metabolites</td>
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<td>FFT</td>
<td>fast Fourier transformation</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<tr>
<td>GGT</td>
<td>gamma glutamyl-transferase</td>
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<tr>
<td>GLDH</td>
<td>glutamate dehydrogenase</td>
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<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<tr>
<td>ION</td>
<td>inferior olivary nucleus</td>
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<tr>
<td>IP</td>
<td>intraperitoneal</td>
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<tr>
<td>IR</td>
<td>immunoreactivity</td>
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KBr  potassium bromide
LBW  live body weight
MCHC  mean cell haemoglobin concentration
MNT  mechanosensory nociceptive threshold
NaCl  sodium chloride
NHS  normal horse serum
NOR  novel object recognition
PB  phosphate buffer
PBS  phosphate buffered saline
PBT-X  phosphate buffered Triton X
PCV  packed cell volume
PFA  paraformaldehyde
PGRS  perennial ryegrass staggers
PRGT  perennial ryegrass toxicosis
pTP  plasma total protein
PVN  paraventricular nuclei
RMS  Root-mean-square
$\tau_{1/2}$  Terminal half life
TMPR  Tremor-motion power ratio
USG  urine specific gravity
UV  ultra violet
VDL  Veterinary Diagnostic Laboratory
Abstract

This thesis evaluates the therapeutic efficacy of potassium bromide within murine and ovine models of Perennial Ryegrass Toxicosis (PRGT). PRGT is a clinical syndrome of herbivores in southern regions of Australia and New Zealand grazing pasture with a high proportion of perennial ryegrass. Peak times of disease are typically late spring and late summer/early autumn. To date no clinically applicable therapy has been available to treat clinical cases of PRGT or to prevent the disease.

PRGT, is a complex toxicity with multiple alkaloids involved and disease ranging from subclinical productivity losses to a severe neurological syndrome with ataxia, tremor, recumbency and occasionally death. Lolitrem B, the primary toxin responsible for neurological signs with PRGT, is thought to block calcium activated potassium channels (BK Channels). In the brain this will have a number of effects; generally it will make neurons unstable or hyperactive. In the cerebellum however the effect is to reduce nerve outputs to other parts of the brain. As the cerebellum is involved in regulating movement the effect of intoxication is for movement to become less regulated and more exaggerated (cerebellar ataxia).

This thesis considered the use of bromide, an accepted therapy for epilepsy in animals and humans, as a treatment for PRGT. Although the mechanism of action has not been fully explained, it is generally accepted that bromide enters brain cells through chloride channels and thereby exerts an inhibitory effect on nerve firing. Bromide could be considered likely to have a non-specific action against the neuronal instability created by BK channel blockade.

Trials in this thesis demonstrate that bromide is effective at reducing tremor and ataxia. Because of its limited side effects, high oral bioavailability, high safety margin and low cost, bromide is a good potential on-farm therapy for PRGT.
Chapter 1 INTRODUCTION

The primary aim of this thesis was to identify a therapy for on-farm treatment of Perennial Ryegrass Toxicosis (PRGT) in sheep. To achieve this, several important areas of research were required prior to therapeutic testing. This included investigation of a naturally occurring outbreak of PRGT to better characterise disease aetiology within a context of farming systems prone to PRGT and the development of suitable testing platforms (both murine and ovine) that could be used to objectively test therapeutic agents.

The success of bromide therapy within the murine model of PRGT (Chapter 5) drove the focus of this thesis to further investigate and test this therapeutic specifically, however the reader should not overlook that within the studies included in this thesis are two research platforms suitable for further investigation of PRGT intoxication and assessment of other therapeutic agents.

A note on nomenclature: within this thesis the term Perennial Ryegrass Toxicosis (PRGT) has been used for the syndrome induced by consumption of high levels of alkaloids in perennial ryegrass (*Lolium perenne* L.) infected with *Epichloë festucae var. lolii* (Reed et al. 2005). This term is preferred to Perennial Ryegrass Staggers (PGRS) as PRGT is commonly used to recognise that the syndrome affecting animals is a complex multi-toxicity with possible synergistic effects of different toxins and not just a neurological disease caused by one toxin. Even though this thesis focuses primarily on the neurological syndrome the author considers that, in a situation where a complex mix of ergot-alkaloids and indole-alkaloids are being consumed by grazing animals and the level of synergism is unknown, arbitrary attribution of effects to one toxin is often not justified. Where lolitrem B has been used as an isolated toxin the term lolitrem B intoxication was used.

PhD candidature was part time for the duration of this thesis.
STRUCTURE OF THESIS

Chapter 2 Literature Review

As the subject matter of this thesis was both specific, in that it was the investigation of a therapeutic agent for PRGT, and very broad, in that no previously developed model of PRGT was suitable for therapeutic testing, likewise then, this literature review needed to cover a broad range of subject material. The format of the literature review is as follows:

Section 2.1 is an overview of the current state of knowledge regarding the aetiopathogenesis of PRGT and potassium bromide (KBr) as a potential therapeutic.

Section 2.2 discusses estimation of impacts on-farming systems in terms of financial losses with an emphasis on the regional economic importance of PRGT.

Section 2.3 discusses the development of knowledge regarding the clinical syndrome, PRGT. It includes records from the earliest observations through to a more detailed understanding of the neurological syndrome (more detail regarding neurological disease is also given in discussion of disease models).

Section 2.4 is a discussion on the development of understanding regarding histopathological lesions.

Section 2.5 is a discussion on the identification of the fungal endophyte, *Epichloë festucae var. lolii*, and its association with clinical presentations of PRGT and then later the identification of lolitrem B as the primary alkaloid involved in causing neurological signs. This section also touches on the potential role of other alkaloids in sub-clinical production losses.

Section 2.6 discusses the development of knowledge regarding the pathophysiological effects of the alkaloids involved in PRGT.

Section 2.7 examines both field and laboratory models used to investigate PRGT. This section also discusses a number of other testing modalities that may be useful in developing models for investigating PRGT, with special consideration of what modalities may be useful for testing therapeutic efficacy.
Section 2.8 discusses potential therapeutics for PRGT. A strong focus was on bromide, its other uses in animals and humans and pharmacokinetic properties. However other potential therapeutics are also discussed.

Section 2.9 is a summary of the current state of knowledge regarding therapeutics for PRGT and discusses the research objectives of this thesis.

Chapter 3 Clinicopathological mechanisms and presentation of perennial ryegrass toxicosis in Australia.

The aim of this Chapter was to improve the understanding of disease aetiology and clinical expression by studying a naturally occurring outbreak of PRGT. Chapter 3 was based on clinical data collected during the 2011 outbreak of PRGT in south-western Victoria. The study forms an important part of this thesis as clinical findings, including presentation of neurological signs and the presence of dehydration, are integral to subsequent research. In particular, two later Chapters (Chapters 6 & 7) investigate the impact of water intake, dehydration and consequent gait analysis in a laboratory model of the PRGT model.

The observation and classification of Type 1/ Type 2 gait changes in this clinical study also forms the basis of later analysis (Chapter 7) where set clinical criteria, based on gait changes, are used to define a time point at which therapeutic intervention is to take place.

The clinical study recorded in Chapter 3 was the first time significant clinicopathological data was collected from field cases of PRGT. By collating pasture, faecal and tissue lolitrem B levels with histopathological data this paper reduces the controversy surrounding the histopathological diagnosis of PRGT.

Funding for this Chapter was supplied by a Technical Assistance Scholarship from Meat and Livestock Australia.

Chapter 3 was published as a paper in the Australian Veterinary Journal:

Chapter 4  Characterisation of neurological dysfunction induced by the BK channel antagonists paxilline and lolitrem B using a mouse model.

Chapter 4 was the first of two Chapters in this thesis based on rodent models of PRGT. Both Chapters examined the neurological effects of the lolitrem B component of PRGT.

Chapter 4 has two aims:

1. To improve the understanding of the neurological effects of lolitrem B and
2. To build a murine PRGT disease model platform for future therapeutic testing.

Chapter 4 asked three key questions, can lolitrem B induced tremor be better characterised with an objective tremor measurement? Secondly, what was the nature of lolitrem B induced coordination defects? The third question comes from clinical observations in Chapter 3, as well as literature reports of behavioural changes (hyperaesthesia, allodynia, aggression, mass drowning events) that imply cognitive deficits in PRGT animals. As a consequence Chapter 3 examined the cognitive effects of lolitrem B using novel object recognition (visual recognition and contextual memory) and the Barnes Maze (spatial learning and memory). As lolitrem B is a relatively novel compound for this type of experiment a positive control was also used in the testing; the well characterised BK channel blocker, paxilline.

Funding for this Chapter was supplied by a Technical Assistance Scholarship from Meat and Livestock Australia.

This Chapter has been accepted for publication in the journal Toxicon.


Chapter 5  Bromide Reduces Tremor and Decreases Perception of Stress after a Single Exposure to Lolitrem B in Mice

The aim of Chapter 5 was to test the hypothesis that potassium bromide (KBr) is an effective therapeutic for reducing the neurological effects of lolitrem B intoxication in mice. The study utilised techniques developed in Chapter 4 for assessment of tremor,
coordination and voluntary motor activity to assess efficacy of therapy. Additionally, forebrain c-Fos expression was assessed as a marker both of intoxication and its alleviation by KBr.

Results from this research provided data for Patent no. US15/038,312; AU2014353885A, Combs M, Quinn J, Edwards S. Prevention and treatment of toxicosis. (Charles Sturt University, James Hagan Court, Boorooma Street, Wagga Wagga New South Wales 2678, Australia) 2015


And

Provisional Patent no. 15/038,261; AU2013904516 Combs M, Quinn J, Edwards S. Stress management in livestock. (Charles Sturt University, James Hagan Court, Boorooma Street, Wagga Wagga, New South Wales, 2678, Australia) 2015


Chapter 6  Development of a Model for Investigation of Perennial Ryegrass Toxicosis in Sheep

Following the successful identification of bromide as a potential therapeutic for PRGT (Chapter 5), using the murine model of PRGT (developed in Chapter 4), the focus of studies moved to testing in sheep. Field testing was not possible due to the unpredictable nature of disease outbreaks in affected areas and the difficulty of objectively assessing effect in this setting. Therefore the aim of Chapter 6 was to develop an induced disease model in a grazing species for therapeutic testing. This chapter described the development of a pen-fed laboratory model of PRGT using the most severely affected grazing species, namely sheep. A particular emphasis of this chapter was the development of a series of objective tests that could be used to grade the severity of disease. Selection of potential testing modalities was based both on observations made, in Chapter 3, 4 and 5, and also an examination of the literature (Chapter 2). From these sources faecal cortisol metabolites, mechanosensory nociceptive threshold, surface electromyography, gait analysis, physical examination, neuro-histopathology, water intake measurement and clinical pathology testing were selected as possible objective testing modalities. This
testing also supported an important additional aim of this study: to improve understanding of the aetiopathogenesis of PRGT.

Funding for this Chapter was provided by Meat and Livestock Australia grant no. B.AHE.0233 “Treatment of clinical signs of perennial ryegrass toxicosis in sheep”.

This Chapter has now been published in the New Zealand Veterinary Journal.


**Chapter 7  Treatment with potassium bromide mitigates ataxia and reduces tremor in lambs with perennial ryegrass toxicosis**

The preceding chapter in this thesis dealt with the investigations into the development of a model that could be used to improve the understanding of the aetiopathogenesis of PRGT in sheep and to provide a platform for future testing of therapeutic agents. In this chapter the aim was to assess the therapeutic agent potassium bromide (KBr) as a treatment for PRGT.

There are two main treatment groups in this study, a group that receives daily KBr prophylactically and a group that receives a single dose of KBr after meeting criteria for a specific clinical stage of intoxication. Assessment of surface electromyograms over the triceps muscle forms an essential objective measure of response to therapy, alongside a composite scale of gait assessment, faecal cortisol levels and histopathological examination.

Funding for this chapter was provided by Meat and Livestock Australia grant no. B.AHE.0233 “Treatment of clinical signs of perennial ryegrass toxicosis in sheep”.

This paper has been published in the New Zealand Veterinary Journal.

Chapter 1  Introduction

Patent no. US15/038,312; AU2014353885A, **Combs M**, Quinn J, Edwards S. Prevention and treatment of toxicosis. (Charles Sturt University, James Hagan Court, Boorooma Street, Wagga Wagga New South Wales 2678, Australia) 2015


And

Provisional Patent no. 15/038,261; AU2013904516 **Combs M**, Quinn J, Edwards S. Stress management in livestock. (Charles Sturt University, James Hagan Court, Boorooma Street, Wagga Wagga, New South Wales, 2678, Australia) 2015


Chapter 8  Discussion

This chapter discussed the contribution this thesis makes to the state of knowledge regarding PRGT, PRGT research platforms and PRGT therapy as well as comparative disease implications arising for the studies described. Potential future directions of research were also discussed.
Chapter 2 LITERATURE REVIEW

2.1 PERENNIAL RYEGRASS TOXICOSIS, LOLITREM B AND THE INVESTIGATION OF THERAPEUTIC AGENTS, INCLUDING BROMIDE

Plant toxicoses cause major problems for cattle and sheep producers in New Zealand and Australia and can result in significant economic loss in production animal industries annually (Sackett and Francis 2006; Sackett et al. 2006). Perennial Ryegrass Toxicosis (PRGT) is caused by a variety of toxins produced by the fungal endophyte, *Epichloë festucae* var. *lolii* (previously *Neotyphodium lolii*) in animals grazing on endophyte-infested perennial ryegrass (*Lolium perenne*) under certain environmental conditions (Gallagher et al. 1981; Gallagher et al. 1982a; Gallagher et al. 1984). The indole diterpenoid, lolitrem B, a potent tremorgenic neurotoxin, plays a key role in the clinical presentation of PRGT. When lolitrem B reaches critical levels in pasture (>1.8ppm), a clinical syndrome, which consists of mild to severe neurological signs, presents in affected flocks and herds (Gallagher et al. 1982b; Cheeke 1995; Combs et al. 2014).

Lolitrem B is a potent tremorgenic neurotoxin that is thought to exert its neurological effects via blockade of calcium activated potassium channels (BK channels) (Imlach et al. 2008; Imlach et al. 2011). BK channels are widely distributed in the central nervous system but are found in high levels within the cerebellar molecular layer and within Purkinje neurons as well as in the vestibular system and deep cerebellar nuclei (Sausbier et al. 2006; Kaufmann et al. 2009). Within Purkinje neurons, BK channels play an important role in the after-hyperpolarisation of spontaneous action potentials and regulating neuronal excitability (Womack and Khodakhah 2002; Kaufmann et al. 2009; Womack et al. 2009). Blockade of BK channels reduces after-hyperpolarisation, increasing neuronal excitability (N’Gouemo 2011). In Purkinje neurons however BK channels are critical for rapid firing spontaneous depolarisation (Raman and Bean 1999). In the Purkinje neurons of mice lacking BK channels a depolarisation-induced inactivation occurs with decreased Purkinje cell firing (Sausbier et al. 2004). Additionally the application of penitrem A (another BK channel blocker) to the axons of Purkinje neurons has been shown to increase the failure rate of high frequency antidromic action potentials and also a decreased inhibitory synaptic response in the deep cerebellar nuclei.
Combined, these findings suggest that a lolitrem B induced BK blockade would decrease cerebellar inhibitory output resulting in the cerebellar ataxia typically observed in clinical cases of PRGT (Sausbier et al. 2004; Johnstone et al. 2012; Hirono et al. 2015).

Typical clinical signs of PRGT include head tremor and ataxia that is exacerbated by exercise (Cheeke 1995) with spinovestibulocerebellar signs noted, including eye deviation (Mayhew 2008). Others have observed that the movement disorder associated with PRGT represents a specific sequence of dyskinesia (Combs et al. 2014). Behavioural changes such as erratic or aggressive behaviour, hyperaesthesia or allodynia have also been noted with PRGT by some authors (Johnstone 2010; Combs et al. 2014; Combs et al. 2018a). A particular feature of PRGT in Australia is the occasional occurrence of high mortality (Reed et al. 2002; Reed et al. 2005; Reed et al. 2011b). Other clinical signs include ill thrift and diarrhoea, with ergot alkaloids possibly playing an important synergistic role in production losses through induction of hyperthermia and reductions in feed intake; this is often referred to as “subclinical PRGT” (Cunningham and Hartley 1959; Di Menna et al. 1992; Cheeke 1995; Reed et al. 2010; Reed et al. 2011a; Fletcher et al. 2017). Ergovaline is the principle ergot alkaloid involved in PRGT, however as noted by Reed et al. (2016) a wide range of ergot alkaloids may collectively contribute to the clinical syndrome.

Currently there are no effective on-farm treatments for clinical outbreaks of PRGT. The sporadic nature of the condition makes field testing of therapeutic agents problematic (Reed et al. 2011a; Fletcher et al. 2017). Previous large animal feed models, feeding 1.4-3ppm DM lolitrem B, have yielded important neurological and pathophysiological information regarding PRGT (Blythe et al. 2007; Johnstone 2010). However, the studies lack objective measures of tremor and the variable progression of signs of intoxication are problematic when trying to apply a therapeutic at a set clinical stage of disease (to ascertain efficacy). This lack of a reliable model has historically impeded the development of regimens for therapeutic intervention and alleviation of PRGT.

The use of bromide in mammals to treat neuronal excitability was first reported in 1876 and is now common, particularly for treatment of idiopathic epilepsy in dogs, although bromide therapy in sheep is previously unreported (Kluger et al. 2009; Baird-Heinz et al. 2018a).
As a simple ion, bromide is taken across the cell membrane through the same calcium-dependent ion channels that generate chloride gradients (Geck and Heinz 1986; Alvarez-Leefmans et al. 1988; Podell and Fenner 1993; Rocha-Gonzalez et al. 2008), the role of which is to generate a high level of intracellular chloride thus hyperpolarising the cell (Alvarez-Leefmans et al. 1988). In vitro bromide has a greater hyperpolarising effect on neurons than chloride and enhances GABA–activated currents resulting in generalised neuronal inhibition (Podell and Fenner 1993; Suzuki et al. 1994; Meierkord et al. 2000).

With regards to bromide’s effect on cerebellar activity, high levels of GABA receptors are found within the cerebellum with GABA(B) expressed at higher levels in the molecular layer (on Purkinje dendrites) than any other region of the central nervous system and GABA(A) expressed at higher levels in the granular layer than any other region bar the frontal cortex (Bowery et al. 1987; Somogyi et al. 1989). The presence of such high levels of GABA receptors in the cerebellum suggest that bromide may reduce afferent inputs to Purkinje neurons and stabilise Purkinje resting membrane potentials.

Currently no effective treatment exists for PRGT in livestock. Although a number of approaches have been evaluated to mitigate the clinical signs of PRGT, such as utilisation of toxin binding agents and magnesium supplements (McColl and Orchard 1981; Allsop and Watters 1984; Reed et al. 2010). There have been some indications of efficacy for mycotoxin binding agents (Reed et al. 2011a), but 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 varieties of perennial ryegrass is a viable option for disease mitigation however pasture renovation is not suitable or economically viable in many areas of Australia and New Zealand. A viable therapeutic that could be delivered at a flock-wide level would be an important addition to the toolbox of mitigation strategies for this disease.

2.2 Estimations of Economic Losses and Farming Impacts.

Graziers in Southern Australia, New Zealand and Tasmania will have frequent outbreaks of PRGT when seasonal conditions support toxin elaboration (Cunningham et al. 1993; Reed et al. 2011c). With the intensity of livestock production in Southern Australia and New Zealand the potential economic impact of PRGT is great. The annual economic loss associated with PRGT in Australia was estimated to be $63.3 million per annum for sheep.
farmers and $8.7 million in cattle production (Sackett and Francis 2006). However this estimate does not take into account potential subclinical losses. Fletcher et al. (1999) noted the difficulty in accounting for the size of subclinical production losses, as well as peripheral effects of disease such as the inability to perform animal husbandry procedures such as crutching or administering anthelmintics due to being unable to move animals. As such, the economic impacts of subclinical production losses as well as compromised parasite control may have a significant impact on-farming systems. Several districts in Victoria and Tasmania suffered severe outbreaks with significant stock losses in 1985-1986, 1992-1993, and 2004-2005, as the conditions were favorable to endophyte growth (Reed et al. 2011c). In 2002 the estimated mortalities numbered in the tens of thousands (Reed et al. 2011c).

2.3 UNDERSTANDING THE CLINICAL SYNDROME: PERENNIAL RYEGRASS TOXICOSIS

2.3.1 Use of ryegrass cultivars and reports of PRGT from the 19th century.

PRGT has a long history in Australia and New Zealand. Perennial ryegrass cultivars were introduced to New Zealand and Australia during the 19th century, mainly from British cultivars (Kirk 1870; Esler and Astridge 1987; Reed 1996). Perennial ryegrass is now grown in an estimated six million hectares in Australia (Cunningham et al. 1994), generally in rainfall regions greater than 550mm per annum (Cunningham et al. 1994) and at latitude 21-44° south (Reed 1996). In Australia the most common cultivar is ‘Victorian’, a wild type endophyte cultivar, which has a relatively low dry matter yield when compared to other cultivars, which reflects its selection based on persistence (Cunningham et al. 1994). This selection method may also account for its association with PRGT, as alkaloid production is associated with pest resistance and drought tolerance (Prestidge et al. 1985; Dowd et al. 1990; Bush et al. 1997; Lane et al. 2000; di Menna et al. 2012). Australasian cultivars in contrast to European cultivars have high levels of endophyte infection (Reed et al. 2000; Soto-Barajas et al. 2013).

2.3.2 Clinical Observations 1880-1959

A disease of farm animals showing similar clinical signs to those now commonly attributed to PRGT was first reported in the New Zealand Country Journal (Anonymous 1880) with the first published report in the veterinary literature appearing in 1906 (Gilruth
Gilruth (1906) described a condition in which muscular incoordination was associated with animals grazing ryegrass, particularly in summer and autumn. Cunningham and Hartley (1959) provide extensive observations of the clinical disease in several species as well as undertaking preliminary feeding and treatment trials. They describe a progression in severity of signs from increases in muscle tone post exercise, to wide based stance and “reeling, drunken” gait through to paresis or even recumbency and death. None of their trialed treatments, which included a number of vitamin and mineral supplements were successful, however the feeding trials did provide strong evidence that a substance in the ryegrass was responsible for the staggers.

2.4 Histopathological Lesions Associated with PRGT (1959-2008)

Histopathological changes were first described by Cunningham and Hartley (1959) and Clegg and Watson (1960) who noted primarily muscular necrosis. Munday and Mason (1967) found muscle necrosis as well, however they also noted in some animals degeneration of cerebellar Purkinje neurons. Eosinophilic proximal axonal bodies associated with the Purkinje cells were the most notable feature of this degeneration although dendritic swelling and central chromatolysis were also noted (Munday and Mason 1967). These features remain central to a histopathological diagnosis of PGRT today, although the significance of the proximal axonal bodies is often disputed as these lesions can be found in older sheep with and without neurological disease (Mason 1968; Bourke et al. 2008).
Figure 2-1: Histological section (H&E) of the cerebellum from a sheep affected by perennial ryegrass toxicosis (PRGT). Histological changes that are characteristic of PRGT include axonal swelling of Purkinje neurons (P) within the granule cell layer (*). Scale bar = 100 μm.

2.5 UNDERSTANDING THE PLANT-FUNGUS-TOXIN INTERACTION

2.5.1 Identification of association with *Epichloë festucae* var. *lolii* infested perennial rye grass (*Lolium perenne* L.) and PRGT.

Observation of systemic infection of perennial ryegrass with an endophytic fungus date back to the 1920’s (McLennan 1920; McLennan 1926) with perhaps the best description in the beautifully written submission to the British Mycological Society by Sampson (1935). In this article Kathleen Sampson notes the potential relationship between the endophytic fungus and toxicity, a suggestion later supported by Neill (1940). However Cunningham and Hartley (1959) dismissed the idea despite identifying the *Lolium* endophyte in most samples analysed stating, “a widely distributed fungus such as the *Lolium* endophyte could not be responsible for localised outbreaks of ryegrass staggers”.

13
Figure 2-2: A scanning electron microscope image of *Epichloë festucae* var. *lolii* mycelium growing on the surface of a perennial ryegrass leaf (*Lolium perenne* L.) (Sealox 2018).

In 1969 elevated alkaloid production in perennial ryegrass was recognised as a potential source of tremor in PRGT (Aasen *et al.* 1969) and tremorgenic mycotoxins were identified as products of a number of fungi belonging to the genera *Aspergillus*, *Balanisa*, *Penicillium*, *Claviceps*, and *Neotyphodium* (Cole *et al.* 1974; Cole and Cox 1981; Powell and Petroski 1993). Gallagher *et al.* (1977) noted that a number of fungal tremorgens produce clinical signs indistinguishable from the naturally occurring clinical syndrome. They also isolated a number of *Penicillium* sp. from toxic cultivars which they cultured.
and fed to sheep with the production of clinical signs, however the toxins produced in culture were not detectable in natural pasture (Gallagher et al. 1977). It was not until 1981 that the association was made between the Lolium endophyte, presence of alkaloid in the plant and the manifestation of neurological signs (Fletcher and Harvey 1981). In the same year Gallagher et al. (1981) identified a number of indole-diterpinoid alkaloids as potential toxins causing PRGT, of these, lolitrem B predominated. Neotyphodium lolii could not be cultured but it was demonstrated that endophyte free perennial ryegrass was also lolitrem free, identifying the source of the toxin as endophytic fungi Neotyphodium lolii (Gallagher et al. 1982a). Subsequent studies were able to purify lolitrem B and through a number of studies identify that it was the tremorgenic compound involved in PRGT (Gallagher et al. 1982b; Gallagher and Hawkes 1986; Munday-Finch 1997).

2.5.2 Identification of lolitrem B toxin as primary toxic agent

Following work identifying increased alkaloids in pasture inducing staggers (Aasen et al. 1969) a strong focus developed looking at tremorgenic fungal alkaloids as a possible source of staggers. The alkaloids initially considered were produced largely from cultures of Penicillium sp. isolated from herbage samples taken at sites of PRGT outbreaks (di Menna et al. 1976; Gallagher et al. 1977; Di Menna and Mantle 1978). Interestingly one family of toxins identified was the janthitrems which would form the key part of the efficacy of AR37 novel endophyte pastures (Gallagher et al. 1980; Finch et al. 2007a; Fletcher and Sutherland 2009). However it became clear that these toxins could not be identified in the plant samples themselves (Gallagher et al. 1977). Another approach was needed and researchers began looking at alkaloid extracts from the pasture samples themselves to identify candidate alkaloids. A critical development of Gallagher et al. (1981) was the extraction of two alkaloids, named lolitrem A and B, from a number of pasture samples.

These alkaloids were indole diterpenoids structurally similar to, but unique from other known tremorgenic mycotoxins (Gallagher et al. 1984). Furthermore they demonstrate some unique clinical features when injected into mice, specifically a slow onset of action and a prolonged tremorgenic activity (Gallagher et al. 1981; Gallagher and Hawkes 1986). Subsequently sheep fed seed containing lolitrem alkaloids were demonstrated to develop staggers after 14 to 32 days on the feed, whereas animals eating lolitrem free seed did not develop staggers (Gallagher et al. 1982b).
Further investigations demonstrated both fungal hyphae and that concentrations of lolitrem B from affected pastures in northern California (Galey et al. 1991; di Menna et al. 1992) also equated with the incidence and severity of PRGT. It was noted that lolitrem B levels in winter were typically below 1µg/kg DM, when the incidence of PRGT was not reported, and that levels over 2 µg/kg DM in spring or summer were commonly associated with clinical signs.

A study of endophyte infected ryegrass pastures in Australia identified Lolitrem B concentrations of up to 6mg/kg (Reed et al. 2000). Toxicity is typically clinically evident at 1.8mg/kg dry matter (although toxicity with lower pasture levels has been recorded) and with high concentrations of alkaloid having a significant impact on animal productivity (Bluett et al. 2001; Bluett et al. 2005; Combs et al. 2014).

2.6 UNDERSTANDING TOXIN-ANIMAL INTERACTIONS

2.6.1 Mechanism of action of lolitrem B toxin

Lolitrem B, produced by the Neotyphodium lolii endophyte, is the indole-diterpenoid alkaloid that is responsible for the neurological signs associated with PRGT (Van Heeswijck and McDonald 1992; Ball et al. 1997; Hovermale and Craig 2001). Perennial ryegrass (Lolium perenne L.) infected with Neotyphodium lolii has improved drought and
pest resistance; however under certain climatic conditions the endophyte will produce significant amounts of Lolitrem endotoxins. The endotoxin lolitrem B is of particular importance to animal production (Mayhew 2008).

Lolitrem B acts on the central nervous system by inhibiting calcium activated potassium (BK) channels, inducing the clinical signs of tremor (Dalziel et al. 2005; Imlach et al. 2008; Mayhew 2008; Imlach et al. 2009). Many tremorgenic mycotoxins induce cerebellar ataxia and a number have been identified as calcium activated potassium channel (BK) inhibitors (Knaus et al. 1994; Cavanagh et al. 1998; Asano and Dick 2011; Imlach et al. 2011). Furthermore BK channels occur with high density in Purkinje cell dendrites and proximal axons and transgenic BK−/− mice show signs of cerebellar ataxia similar to that seen with lolitrem B intoxication (Kaufmann et al. 2009; Sausbier et al. 2004; Sausbier et al. 2006). Imlach et al. (2008) demonstrated, using BK−/− mice and human embryonic kidney cells expressing high levels of BK channels, that lolitrem B appears to be a potent inhibitor of BK channels.

There is an abundance of BK channels within both the forebrain and the cerebellum, the distribution intensity of these channels however is varied, both throughout the brain and within the individual neurons (Sailer et al. 2006; Sausbier et al. 2006; Piwonska et al. 2008; Kaufmann et al. 2009). Both distributions will affect the pathophysiology of BK channel blockage although the relationship between the toxin, the extent of the blockade and the neuronal response is complex and poorly understood. Purkinje cells are the major output neuron population of the cerebellum, synapsing with neurons in the deep cerebellar nuclei, which in turn synapse, with various pre-motor centres in the brain to coordinate movement (Purves et al. 2001). As these cells express high levels of BK channels, inhibition of BK channels in these neurons will reduce the ability of these neurons to repolarise. BK channel blockade has been demonstrated to reduce after-hyperpolarisation and drastically reduce spontaneous bursting action potentials from Purkinje cells, in effect switching the cell’s neuronal conduction off (Sausbier et al. 2004; Cheron et al. 2009).

BK channels are also expressed at high levels in the deep cerebellar nuclei and many other regions of the brain including reaching high levels in the dentate gyrus of the hippocampus (memory, stress regulation and spatial behaviour), as well as the substantia nigra pars reticulate and the entopeduncular nucleus, both major GABAergic output
nuclei (Deniau and Chevalier 1985; Chevalier and Deniau 1990; Parent et al. 1999). The substantia nigra pars reticulate in primates also plays an important role in control of eye movements (Sausbier et al. 2006). Therefore with increasing severity of intoxication a broader range of neurological signs will be expressed (Combs et al. 2014). In PRGT-affected livestock the extent of ataxia produced may therefore be determined by the extent of BK channel blockade in the cerebellum with a mild blockade producing problems with pre-motor sequencing and initiation of movement and more severe blockade producing dystonia, myoclonus and “cerebellar seizures”.

BK channels are not only present in neural tissue but also in other organ systems in the body, in particular, vascular, renal, and gastrointestinal tissues (Smith et al. 1997; Pluznick and Sansom 2006; Wulf et al. 2009). The extent to which the wide distribution of BK channels is associated with the subclinical losses associated with PRGT is unclear. However scours is a commonly observed clinical sign of PRGT and lolitrem B intoxication has been demonstrated to affect ruminal motility (Bluett et al. 2001; Tor-Agbidye et al. 2001; Wang et al. 2003). The toxin increases the intrinsic rate of smooth muscle contraction of the reticulorumen (Wang et al. 2003; Gupta 2007) as well as affecting heart rate, respiration rate, vascular tone and blood pressure (Henry et al. 2016b; Henry et al. 2016a; Imlach et al. 2010; Dalziel et al. 2005; McLeay and Smith 1999).

Lolitrem B however is not the sole alkaloid produced by *N. lolii*; the ergot alkaloid ergovaline is also produced in significant quantities. There have been varying reports on the levels of ergovaline in perennial ryegrass pasture, some suggesting that it reaches clinically significant levels (Reed et al. 2011b) and others suggesting it rarely does so or lacks clinical significance (Hovermale and Craig 2001; Finch et al. 2018). However the absolute levels of ergovaline and lolitrem B may be less significant than the possibility of synergistic activity, with both toxins having neural effects, neuro-hormonal effects and causing peripheral vasoconstriction (Dyer 1993; Larson et al. 1999; Cheong et al. 2002; Sausbier et al. 2005; Holtzclaw et al. 2011; Alabdouli et al. 2013; Oliver 2008).

2.6.2 **Subclinical production losses and the role of other fungal alkaloids in PRGT.**

The realisation that subclinical production losses formed an important part of the disease process came relatively recently in the understanding of the pathological processes surrounding PRGT. Fletcher (1982) had originally noted that sheep grazing cultivars
associated with high rates of PRGT did not have reduced growth rates compared to flocks presenting with a low incidence of disease. However in a later study it was found that low endophyte pasture was severely affected by pests which could affect pasture growth and subsequently live weight gain and that animals grazing pasture with a high level of endophyte did have decreased growth rate compared to animals grazing low/ no endophyte pasture, if pasture growth was similar (Fletcher 1986). Later still it was noted that clinical signs of general ill thrift, loosely classified by farmers as “autumn or summer ill thrift”, namely high rates of dags, scouring and poor weight gain/condition, did not occur in animals grazing endophyte free pastures (Fletcher and Sutherland 1993; Fletcher et al. 1999).

In recent years a growing focus of research around subclinical disease has considered the role of other alkaloids present in perennial ryegrass cultivars that may have a synergistic action or be responsible for subclinical toxicity. Ball et al. (1995) looked at the inter-relationship between peramine and lolitrem B and it’s associations with fungal endophyte concentration. They noted similar seasonal fluctuations in alkaloid levels to other studies and a strong association with endophyte level, with relatively higher levels of lolitrem B in the base of the plant and peramine in the regrowth portion of the plant. This is significant because eating down into the crown of the plant has been associated with development of PRGT (Tor-Agbidye et al. 2001).

Hovermale and Craig (2001) analysed 459 ryegrass straw samples and noted a strong correlation between lolitrem B levels and ergovaline levels although noting that ergovaline typically did not come within the accepted range for toxicity. However, other authors have demonstrated a synergistic effect on smooth muscle activity between lolitrem B and ergot alkaloids, with the combination of both toxins increasing smooth muscle contraction beyond a purely summative increase in contractility (Dalziel et al. 2013). Such an interaction would lower the toxic threshold of both toxins, although understanding the degree of synergism from a clinical perspective has been hampered to date by the difficulty of studying lolitrem B toxicity in the absence of ergot alkaloids in a large animal model.

Hill (2005) suggested that another lysergic acid based compound may play a role in subclinical production losses. Subsequent research has noted that a range of ergot
alkaloids appear to be present in toxicity outbreaks and it may be better to think of total ergot alkaloid contribution to toxicity rather than just ergovaline (Reed et al. 2011b; Reed et al. 2011c; Reed et al. 2016). Reed et al. (2016) also noted that, in Australia, the ratio of ergot alkaloids compared to lolitrem B were relatively high and may play a more significant role in toxicity, particularly in environments with high temperatures.

In short, the aetiological agent responsible for these “subclinical losses” is still debated (Reed et al. 2000; Reed et al. 2016; Fletcher et al. 2017; Finch et al. 2018). Although it is likely that a number of toxins play a role. Both ergovaline and lolitrem B have the potential to cause production losses through hyperthermia (through peripheral vasoconstriction) and gastrointestinal effects (scours with lolitrem B and inappetence with ergovaline) (Smith et al. 1997; McLeay and Smith 1999; McLeay et al. 1999; Aiken et al. 2011; Henry et al. 2016b; Henry et al. 2016a).

Discussions around the relative roles of the numerous alkaloids involved in this syndrome has led some authors to suggest that to account for the role other toxins play in ryegrass toxicity and to recognise that components of the toxicity may produce subclinical production losses, rather than simply tremors, the term perennial ryegrass toxicosis should be preferred to perennial ryegrass staggers (PRGS) (Reed et al. 2011b; Reed et al. 2011c).

2.7 LABORATORY MODELS OF PRGT

Laboratory models of disease form an important link with naturally occurring disease, both in understanding the aetipathogenesis of disease, for identification of targets for drugs and for drug testing (Martin et al. 2005; Imlach et al. 2008; van der Worp et al. 2010; McGonigle and Ruggeri 2014). Although often criticised for poor ability to translate to clinical outcomes in humans, many of these concerns are reduced when, unlike with humans, the same species of animal can be used in disease models as is affected by the naturally occurring disease (McGonigle and Ruggeri 2014). Despite this study the design and validity of model construct can still be of concern in animal models and close consideration of these factors needs to be undertaken to ensure that the findings indicate clinical relevance and even then clinical trials are critical (van der Worp et al. 2010; McGonigle and Ruggeri 2014).
Chapter 2  Literature Review

The development of two animal models for PRGT forms an important part of this thesis; the following section therefore examines previous PRGT models in a range of species.

2.7.1 Sheep Models of Perennial Ryegrass Toxicosis

The first attempts at developing an experimental model in sheep for examining perennial ryegrass toxicosis was undertaken by Keogh (1973) with the modest aim of testing the hypothesis that grazing the crown of perennial ryegrass was more likely to generate toxicity than grazing leaf matter. This study also generated what later became known as the “Keogh Scale” (Table 2-1), providing a scale of clinical severity in sheep which has been commonly used in research to assess PRGT cases since its publication (Fletcher 1986; Blythe et al. 2007; Finch et al. 2011; Fletcher et al. 2017).
Table 2-1: The system of scoring generated by Keogh (1973) used to assess severity of ryegrass staggers clinical signs in sheep, known as ‘The Keogh Scale’.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description of clinical signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Slight trembling of neck, shoulders, and flank muscles after hard exercise (400 m run).</td>
</tr>
<tr>
<td>2</td>
<td>Marked trembling of neck, shoulders and flank muscles, and shaking of head after hard exercise, but no lack of co-ordination.</td>
</tr>
<tr>
<td>3</td>
<td>Marked trembling of general musculature and head shaking; some lack of co-ordination of movement and impaired vision while running.</td>
</tr>
<tr>
<td>4</td>
<td>Muscle tremors and head shaking after a short run (&lt; 30 m) or sudden disturbance; continued exercise elicits a marked lack of coordination resulting in a characteristic staggering gait which normally ends with the animal falling down; a short period of moderate to severe muscular spasm follows, after which the animal is able to regain its feet and walk off.</td>
</tr>
<tr>
<td>5</td>
<td>Severe muscle tremors elicited by slight disturbance or exercise (&lt;10 m rapid movement) which invariably result in staggering and collapse in a severe tetanic spasm which may last up to 20 min in very bad cases.</td>
</tr>
</tbody>
</table>

This scale focuses on the degree of neurological derangement after driving animals, ranging from 1, mild tremor after extensive exercise, to 5, falling and unable to rise after brief movement (Table 2-1). The scale has been widely applied and is useful although it does not separately assess gait abnormalities, cognitive function and tremor meaning it is therefore possible to have animals meeting criteria for different points of the scale. Like all scales based on clinical observation it is inherently subjective and prone to observer experience and bias. There is no evidence that the points on the scale represent a true linear progression of the intoxication. Additionally, the Keogh Scale assumes a linear relationship for distance travelled prior to showing signs and severity of disease without accounting for exercise intensity. For instance in Category 3 there is a note that signs may start either after a short run or a “sudden disturbance”, the later suggesting the animal
cannot coordinate rapid acceleration/ limb movements, however there is no means of accounting for this within the scale.

Following this original study a series of studies were undertaken that focused on the association between clinical signs with the presence of endophytic fungus (Fletcher and Harvey 1981; Fletcher 1982; Fletcher 1986). These papers focus on animal productivity and understanding the clinical manifestations or mechanism of toxicity is not the focus of these studies. As such, the same simple scale of disease severity is alone used; in many ways they resemble pasture trials.

Running parallel to these studies a number of studies attempting to elucidate the nature of the toxin present in perennial ryegrass were being undertaken. These studies were initially greatly hampered by the inability to culture the endophytic fungus, although lolitrem B was eventually identified as a likely candidate toxin (Gallagher et al. 1981; Gallagher et al. 1982b; Gallagher et al. 1984; Gallagher and Hawkes 1986). Importantly, Gallagher et al. (1982b) included a small bioassay study using lolitrem containing seed in animal feed. The focus however again was the association of the toxicity with the presence of the toxin, and a detailed investigation of neurological dysfunction was not undertaken.

These two lines of study (association of fungus and lolitrem toxin with PRGT) then intersected in a study undertaken by di Menna et al. (1992) where fungal hyphae counts, lolitrem B concentrations and incidence of staggers were correlated. However again, no attempt was made to categorise the intoxication beyond the “Keogh Scale”.

With the ability to produce lolitrem B in small amounts Smith et al. (1997) injected toxin into a small number of sheep and performed EMG recordings from the shoulder, neck, pyloric antrum and duodenum. Although the method of analysis of EMG recording is not clear it appears that lolitrem B intoxication caused a prolonged increase in skeletal muscular activity. In smooth muscle there was an initial increase in activity, followed by a prolonged reduction in activity. In a second paper by the same group an increase in EMG recordings from skeletal muscle over the shoulder and an increase in head movement is noted but not quantified (McLeay and Smith 1999). In this study increases in blood pressure and respiration rate, but not temperature, were also noted (McLeay and Smith 1999).
Fletcher et al. (1999) examined respiration rate, body temperature, prolactin levels and dag formation, thereby taking important steps to recognising that PRGT was a multi-toxin intoxication. Fletcher et al. (1999) noted in particular that the ergot-alkaloid ergovaline appeared to play a perhaps synergistic role in production losses.

Following this initial examination of synergistic effects of indole and ergot alkaloids in PRGT, a series of studies focused on the effects of ergot alkaloids on sheep and their potential role in PRGT. McLeay et al. (2002) demonstrated that ergovaline, the prominent ergot alkaloid present in PRGT, induced hyperthermia and hypertension and was of increased potency compared to ergotamine. Neither ergovaline nor ergotamine induced tremors as recorded by EMG in this study (McLeay et al. 2002).

A series of pen-fed laboratory experiments using high ergovaline plus lolitrem B containing feeds demonstrated the capacity of these feeds to increase temperature, respiration rate and oxygen consumption with reduced dry matter intake and liveweight gain (Henry et al. 2007; Henry 2012; Henry et al. 2016b; Henry et al. 2016a). Experiments in controlled temperature environments demonstrated that these changes were also exacerbated by high ambient temperatures making the potential for ergot alkaloids to be critical factors in the disease process as seen in Australia, where high ambient temperatures are common (Reed et al. 2011c; Henry 2012; Combs et al. 2014; Henry et al. 2016a).

These laboratory experiments were supported by a field trial, using a number of novel/wildtype endophytes with varying alkaloid profiles, which confirmed high ergovaline feeds increased the risk of hyperthermia (Fletcher et al. 2017). Interestingly, and in contrast to the laboratory experiments done by Henry et al. (2016a), a production effect was not seen in this trial with any endophyte variety although there was a large inter-year variation in growth rates with animals in the second year of the trial, where high alkaloid concentrations were recorded, as well as poorer growth rates.

Finch et al. (2018) observed no difference in expression of neurological signs of PRGT between novel endophyte AR98, which produces lolitrem B and no ergovaline, and wild type endophyte. The study does not account for other ergot alkaloids or assess animal productivity so it is difficult to make a definitive assessment on any synergistic role of ergot alkaloids in the production of clinical signs or production losses. Reed et al. (2016)
noted a broad range of ergot alkaloids present in some cultivars and postulated that a global assessment of total ergot alkaloids may be more valuable than simply focusing on ergovaline when assessing their role in PRGT.

Overall, there has been little progression in the understanding of the neurological expression of PRGT in sheep since Keogh (1973), with research focusing primarily on production aspects of disease or toxin identification. EMG analysis undertaken by (McLeay et al. 1999) is an important exception and further research to aid quantification of EMG readings may allow this to form a more objective and reliable measure of neurotoxicity. The Keogh Scale is a useful observation technique, however better categorisation of neurological changes within the scale would allow improved assessment of disease severity. Progression of these and other neurological testing are needed if assessment of therapeutics is to be undertaken.

2.7.2 Cattle Models of PRGT

Although numerous clinical observations of PRGT have been reported, most cattle models looking at PRGT, have looked at production effects only, and therefore have little to say about disease pathogenesis or clinical features that could be used as markers for therapeutic trials (Bluett et al. 2005; Fink-Gremmels 2008; Thom et al. 2013). The exception to this is a study by Blythe et al. (2007). In this study 61 cattle were feed ad-lib chopped ryegrass hay at levels of 0-3.058mg/kg DM. The aim of the study is to bioassay the dose at which lolitrem B in feed will produce clinical signs in cattle. Cattle consuming 1.4mg/kg DM did not develop neurological signs, but half of the cattle on the 1.954mg/kg DM feed developed ataxia. All the cattle in the highest dose range (3.058 mg/kg) developed neurological signs. The earliest development of clinical signs was 12 days. Animals had twice daily observations done by blinded observers and rated by a modification of a scoring system (Table 2-2) developed by Galey et al. (1991) for sheep.
Table 2-2: Blythe Scoring System developed to assess severity of ryegrass staggers in cattle (Blythe 2007)

<table>
<thead>
<tr>
<th>Score</th>
<th>Description of clinical signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No clinical signs</td>
</tr>
<tr>
<td>1</td>
<td>No resting tremors or incoordination; moderate-intensity tremors and incoordination with handling.</td>
</tr>
<tr>
<td>2</td>
<td>No resting tremors or incoordination; moderate-intensity tremors and incoordination with handling; marked stiffness of gait.</td>
</tr>
<tr>
<td>3</td>
<td>Spontaneous low-intensity tremors and incoordination at rest; moderate to severe tremors and incoordination with handling; marked stiffness of gait, difficulty in rising</td>
</tr>
<tr>
<td>4</td>
<td>Pronounced resting tremors and incoordination; convulsive tremors and severe incoordination with handling; extreme spastic gait (goose stepping)</td>
</tr>
<tr>
<td>5</td>
<td>Severe spontaneous tremors and incoordination at rest, usually accompanied by collapse into recumbency and convulsive episodes.</td>
</tr>
</tbody>
</table>

The scoring system has several limitations, in particular the terms used for the degree of neurological changes are not clearly defined in the scale (e.g. marked stiffness of gait vs. extreme spastic gait, or severe tremor vs convulsive tremor). The scoring scale is also at odds with that used by Combs et al. (2018b) who observed falling or dropping into sternal recumbency in animals with signs otherwise only rating 2 or 3 on the Galey/Blythe scale. This may reflect a species difference or that active circling of animals induced a greater gait change at a lower level of toxicity. Blythe et al. (2007) also observed proprioceptive deficits which have not been noted by other authors, although the method by which these deficits are examined is not described and what is observed may be poor limb/ body positioning due a combination vestibular and cerebellar ataxias (Schniepp et al. 2017), as postulated by Combs et al. (2018b) and Johnstone et al. (2012) rather than poor limb sensation. The level of lolitrem B in feed (around 1.9mg/kg) at which clinical signs of intoxication are induced is consistent with observations by most other authors (di Menna et al. 1992; Tor-Agibidye et al. 2001), however other authors have noted intoxication may occur at levels as low as 0.97mg/kg (Miyazaki et al. 2000; Miyazaki et al. 2004; Combs et al. 2014). Blythe et al. (2007) also took tissue samples from the animals at the highest
Chapter 2  Literature Review

feed levels and located lolitrem B in fat samples up to 0.061mg/kg fat (wet tissue). Miyazaki et al. (2004) found higher levels of lolitrem B (up to 0.21mg/kg fat) feeding only 1.2mg/kg DM straw over a longer time period. Both groups failed to find lolitrem B in other tissues, highlighting the highly lipophilic nature of lolitrem B.

2.7.3 Equine Model of PRGT

Early observations of horses affected by PRGT include observation of clinical cases by Cunningham and Hartley (1959) who noted a “reeling, drunken gait”, and in some cases a “posterior paralysis”. Given that the author explains that animals typically recover from the later after a few days if moved to new pasture it can be assumed that “posterior paresis” is intended. These clinical signs are similar to those noted by other authors in sheep, where the hindlimb paresis would appear to be due to a combination of weakness (often from dehydration), muscle damage from falling and inability to correctly coordinate extensor muscle groups due to cerebellar/vestibular ataxia (Combs et al. 2011; Combs et al. 2018b; Keogh 1973; Mayhew, 2008).

Perhaps some of the best neurological observations in a large animal model do not come from the major production species but rather in work done by Johnstone (2010) on horses, where detailed assessment of neurological changes were undertaken as well as investigation of auditory and magnetic invoked action potentials and flow mediated potassium excretion. In this study seven horses in two groups were feed either 2.1 or 2.2ppm lolitrem B plus 2.0 or 2.6ppm ergovaline ad libitum for 2 weeks. Horses where clinically examined for gait disturbances by lunging daily. A standardised neurological examination was performed at the start and end of the test period (2 weeks or when the animals reached 3/5 on modified Keogh Scale). Johnstone et al. (2012) noted that the horses’ thoracic limb musculature was predominantly affected by tremor, notably the pectoral and shoulder musculature. The tremor was recorded at approximately 3Hz and was exacerbated by exercise, particularly changes in direction of movement. The tremor was most prominent in the weight bearing limb suggesting that the tremor may have been associated with maintenance of normal body position and regulation of contractile force in extensor muscle groups.

Ataxia was noted as a truncal sway with a wide base stance (Johnstone et al. 2012). Animals also had increased levels of limb placement deficits with increasing toxicity and
these were greatly exacerbated by blindfolding animals and/or complex movements such as tight circling. However no evidence of paresis was found on the tail pull test and animals had normal hopping reflex. No animals fell or became recumbent during testing (Johnstone et al. 2012).

A unique observation in Johnstone et al. (2012) was the presence of a fine, high frequency (~5Hz) nystagmus in six of seven animals in the study. This was identified by direct ophthalmic examination. No other cranial nerve changes were noted. Animals had increased sensitivity to the withers slap test and this was interpreted by the author as hyperaesthesia or allodynia. The authors noted considerable variation in tremor onset and severity. Two horses developed signs of ergot toxicity despite being in the lower ergovaline group, suggesting that other ergot alkaloids, not measured, may also have been present (Johnstone et al. 2012).

A second related study in horses looked at flow-mediated potassium excretion in lolitrem B which demonstrated reductions in flow mediated potassium excretion and reduced aldosterone (Johnstone and Mayhew 2012). The level of aldosterone may have been affected by ergovaline, a dopamine agonist, in feed (Johnstone and Mayhew 2012), highlighting the difficulty of extrapolating the mechanism of action of lolitrem B in the presence of other alkaloids.

2.7.4 Camel Model of PRGT

Alabdouli et al. (2014) conducted a study in camels that had a number of important findings. The study was conducted with similar methodology to the study conducted by (Blythe et al. 2007) with animals fed ryegrass straw ad libitum at four concentrations of lolitrem B (0, 1.111, 1.478 and 2.273 mg/kg DM) for 56 days. Animals were assessed for neurological changes but also a number of physiological parameters were examined and necropsy with histopathological analysis was undertaken in two control animals and two animals from the high toxin groups.

An important observation from this study was that animals from all groups that received toxin appeared to develop neurological signs of intoxication. This represents a considerable reduction in the lolitrem B concentrations in feed needed to induce neurological disease compared to most other studies (di Menna et al. 1992; Tor-Agbidye et al. 1994; Blythe et al. 2007; Alabdouli et al. 2014) although some studies have found
similar lolitrem B levels do induce intoxication (Miyazaki et al. 2000; Miyazaki et al. 2004; Combs et al. 2014). Possible explanations include the potential for high bioavailability of lolitrem B in straw or a species specific sensitivity to the intoxication. Straw has been used in other studies that produced low threshold levels for intoxication and considerable variation between species in apparent in degree of neurological signs, with a similar species (alpacas) appearing to be highly sensitive to disease (Sampaio et al. 2007; Sampaio et al. 2008; Reed et al. 2010; Mackintosh and Orr 1993; Holmes et al. 1999; Miyazaki et al. 2000; Miyazaki et al. 2004; Mouser et al. 2009).

Falling, weakness and ataxia occurred at all feed levels in this study and ataxia was considered a more prominent sign than tremor although some animals in the higher toxin groups did develop a prominent tremor in the thoracic limbs and pectoral muscles, with one animal reaching the highest level of intoxication, using the Galey et al. (1991) modification of the Keogh Scale; that is pronounced resting tremors and incoordination, falling into lateral recumbency and difficulty rising. The authors noted hair loss in this animal and do not account a reason to it, but extended periods of recumbency appear likely.

A dose dependent weight loss was observed in the camels, despite camels having higher protein intake in the higher toxin feed groups (Alabdouli et al. 2014). Increased blood urea nitrogen (BUN) is reported in the study and linked to renal histopathological, although differences in protein intake are another possible explanation of this finding (Preston et al. 1965; Lake et al. 1974). Additionally BUN is sensitive to dehydration and other authors have observed that BK channel blockade may affect renal and bowel function and water intake (McLeay et al. 1999; Combs et al. 2014; Combs et al. 2018b).

Histopathological changes in the cerebellum reported in this study are consistent with other studies (Sampaio et al. 2008; Combs et al. 2018b). Reports of vacuolar changes in the liver may reflect changes seen with a negative nutritional state, likewise renal changes may reflect severe renal pathology relating to poor renal perfusion resulting from shock, but given that these animals had a higher level of toxicity for longer than most animals in other studies a primary renal toxic injury cannot be ruled out.
2.7.5 Rodent Models of PRGT

Rodents have been used primarily as a simple bioassay for toxins isolated from perennial ryegrass or cultures of fungi associated with perennial ryegrass (Gallagher et al. 1980; Gallagher and Hawkes 1986) rather than any attempt to model disease or understand underlying mechanisms. The exception to this is work done using both transgenic (BK<sup>-/-</sup>, β1 and 4 negative) mice and lolitrem B or paxilline intoxicated mice (Imlach et al. 2008). Examining both tremor intensity and duration, using a visual scale, and coordination, using the Rotarod device Imlach et al. (2008) found that lolitrem B had a prolonged duration of effect and slower onset of effect on both parameters when compared to paxilline. Compared to BK<sup>-/-</sup> mice, lolitrem B mice showed more tremor at peak effect, but similar latency to falling. Interestingly, although BK subunit β4<sup>-/-</sup> mice had a similar tremor to wild type (WT) mice when exposed to lolitrem B, they were less ataxic, suggesting that the β4 subunit of BK channels may be an important drug target for reducing ataxia.

Although there has been relatively little research in mice regarding lolitrem B intoxication, there is of course extensive murine models for the study of neurological dysfunction. As this thesis involved the development of a murine PRGT model a focus on further examination of the literature with regards to assessment of tremor, ataxia, spatial learning and memory and c-Fos as a markers of neurological activity is warranted. Details of rodent models follow:

2.7.5.1 Tremorgenic toxins in rodent models

Rodent models have been used previously to assess the tremor characteristics of a number of toxins that induce cerebellar tremor (Cavanagh et al. 1998; Martin et al. 2005; Paterson et al. 2009). Additionally a number of genetic mutants including BK<sup>-/-</sup> and GABA(A) receptor Alpha-1 subunit knockout mice have been used to examine tremor (Sausbier et al. 2004; Miwa 2013).

Harmaline features prominently in rodent studies of cerebellar tremor as it is seen as a useful animal model for essential tremor in humans as both are responsive to ethanol therapy and in both changes in the olivocerebellar system appear to play a prominent role (Martin et al. 2005; Paterson et al. 2009; Miwa 2013). Harmaline is a β-carboline derivative and an inhibitor of monoamine oxidase A (Udenfriend et al. 1958; Kim et al.
1997) although the mechanism of its tremorgenic activity has been postulated to in fact be via binding to the benzodiazepine receptor site on GABA receptors (Robertson 1980). Functionally harmaline acts preferentially on neurons of the inferior olivary nucleus (ION) enhancing rhythm generation (Miwa 2007). Increased activity in the ION then acts on the cerebellum via excitatory projections to the Purkinje neurons (known as climbing fibres). The central role of the ION in the generation of tremor has been demonstrated by resolving tremor by destroying the climbing fibres (Simantov et al. 1976). Harmaline also induces a cerebellar Purkinje neuron degeneration in rats, but not mice, where rather, a microgliosis is induced in the ION (Miwa et al. 2006).

In contrast to harmaline, destruction of the ION does not abolish tremor in rats administered the indole diterpenoid mycotoxin penitrem A, suggesting another mechanism of action (Cavanagh et al. 1998). Pathological changes in penitrem A intoxicated rodents included focal necrosis of the granular layer and pyknosis and vacuolation of Purkinje neurons with extensive Purkinje cell loss (Cavanagh et al. 1998). Lesions were most prominent in the vermal/paravermal regions (Cavanagh et al. 1998). Proximal axonal swelling of Purkinje neurons were noted largely in animals euthanased 42-70 days post exposure (Cavanagh et al. 1998), suggesting this change reflects chronic or delayed pathological processes. These changes reflect similar changes found in lolitrem B intoxicated sheep (Munday and Mason 1967).

Knaus et al. (1994) using bovine aortic smooth muscle cell cultures, identified a number of indole diterpenoid mycotoxins as having high binding affinity for calcium activated potassium (BK) channels. Using patch clamping techniques this binding was shown to be associated with a marked decrease in membrane permeability of potassium (Knaus et al. 1994). These experiments did not include the indole diterpenoid lolitrem B, however similar experiments later confirmed that lolitrem B blocked BK channels (Dalziel et al. 2005). Lolitrem B has also been shown not to effect BK knockout mice (Imlach et al. 2008).

2.7.5.2 Tremor Characterisation in Rodent Models

Tremor has been characterised in rodent models using three broad techniques; visual scaling, electromyography (EMG) and the use of pressure sensors.
Visual scaling has been used extensively in rodents for the assessment of lolitrem B induced tremor (Munday-Finch 1997; Imlach *et al.* 2008), typically using a scale developed by Gallagher and Hawkes (1986) (Table 2-3).

**Table 2-3: Visual rating scale for tremor assessment in rodents (Gallagher and Hawkes 1986)**

<table>
<thead>
<tr>
<th>Rating</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No tremor, animal behaviour normal.</td>
</tr>
<tr>
<td>1</td>
<td>No resting tremor. A short-duration, low-intensity single-burst whole body tremor elicited by exercise/handling.</td>
</tr>
<tr>
<td>2</td>
<td>No resting tremor. Several moderate-intensity whole body tremor bursts elicited by exercise/handling.</td>
</tr>
<tr>
<td>3</td>
<td>Spontaneous, continuous low-intensity resting tremor may be present. Repeated moderate to severe intensity tremor bursts elicited on exercise/handling.</td>
</tr>
<tr>
<td>4</td>
<td>Pronounced, protracted, spontaneous resting tremor. Movement, exercise, or handling may induce convulsive episodes in addition to severe tremor.</td>
</tr>
<tr>
<td>5</td>
<td>Severe spontaneous tremor, usually accompanied by convulsive episodes, and eventually culminating in death.</td>
</tr>
</tbody>
</table>

This rating scale would appear to be based broadly on rating scales used in observation of perennial ryegrass toxicosis in sheep (Keogh 1973; Galey *et al.* 1991). This scale is useful in that it gives something of a global assessment of the tremor in the animal however it has a number of drawbacks. The tremor scale is clearly subjective, although training will improve assessment accuracy. The scale itself contains a number of components; tremor at rest, tremor with movement, seizures and this assumes that these components will always happen in the same sequence with increasing intoxication in every animal, however this is unlikely to be the case. Finally the scale assumes the change between point allocations of severity is linear; however without objective measures to compare the severity of tremor at each point allocation this assumption may not be correct and this will make statistical analysis of this scale difficult. Visual tremor scaling should therefore only be used as an adjunct to other measures of tremor severity. Furthermore some authors have suggested that length of duration of tremor post exposure implies duration of action/half-life of toxin; although this may be the case dysfunction induced
by tissue/physiological damage is also possible and so interpretations of toxin dynamics should be cautiously interpreted where direct measurements of tissue levels and/or histopathological changes have not been assessed.

Cavanagh et al. (1998) performed EMG analysis of penitrem A induced tremor by anaesthetising intoxicated rats and placing 27g recording needles into the dorsal thoracic musculature. Sampling was amplified at a bandwidth of 0.3-5000Hz and fast Fourier transformation (FFT) was used to analyse tremor frequency. FFT analysis demonstrated a tremor peak at around 7Hz, with tremor starting from 7-10 minutes post injection. However the tremor peak dissipated so that by 6 hours it was replaced by increased EMG activity across a broad spectrum of frequencies (Cavanagh et al. 1998). This form of assessment improves objective assessment of tremor peak although as noted tremor peak does dissipate and Root-Mean-Square (RMS) or area under the curve measurements may give a better indicator of the effects of the toxin on tremor. The technique is also highly invasive and restricts natural movement of the animal thereby limiting what other behavioural analysis may be performed on the animal.

A number of forms of pressure sensors have been employed in an attempt to measure power output from muscle contractions associated with tremor. Dill et al. (1968) developed a device that use an electromagnetic coil under an animal container, with pressure from the animal moving a magnet through a copper coil to generate a charge. This basic technique was then improved upon by the use of piezoelectric crystals or ceramic that generate a current when placed under stress (Lehtinen and Gothoni 1985). Lehtinen and Gothoni (1985) highlighted the advantages of this technique over other techniques noting that the animals are not restricted in their movements by the recording device and that highly accurate spectral recordings (tremor frequency) can be made. However these devices have a number of limitations, tremor power output is only recorded in one plane (vertical) whereas tremor occurs in three planes and possibly rotationally (Lehtinen and Gothoni 1985). Also actual power output from the tremor can only be estimated, partly for the above reason but also because the mass of the limbs involved in the tremor and the transfer characteristics of the container the animals are placed in can only be estimated (Lehtinen and Gothoni 1985). Despite this the use of piezoelectric pressure sensors to measure tremor frequency and relative intensity has been
adopted by numerous rodent models of tremor (Gothoni et al. 1983; May 2003; Martin et al. 2005; Martin and Handforth 2006; Paterson et al. 2009).

Martin et al. (2005) made important modifications to the protocol proposed by Lehtinen and Gothoni (1985), increasing the time of measurement epochs and also using a ratio between peak tremors and power output at all motion frequencies as the measure of tremor intensity. The latter is of particular significance with intoxications involving intention tremor as animals that move more tend to demonstrate more tremor, whereas animals that are acutely intoxicated often have very little movement, perhaps as a behavioural response to limit tremor or ataxia (Combs et al. 2019). This model has subsequently been tested with several drugs known to be effective in the treatment of essential tremor and has proven to be effective if differentiating changes in tremor (Martin et al. 2005; Martin and Handforth 2006).

Paterson et al. (2009) attempts another method of analysis by using waveform data to analyse number of tremor events. However it is unclear that this method is effective for all the diversity of tremor events, particularly how tremor events of different length should be quantified is problematic. Also there is no accounting for degree of movement with action induced tremor.

2.7.5.3 Characterisation of Cerebellar Ataxia

A large number of techniques have been used to characterise cerebellar ataxia in mice, including footprint pattern, beam walk, Rotarod and ladder walk (Sausbier et al. 2004; Imlach et al. 2008a; Chen et al. 2010). Of these only the Rotarod has previously been used as a measure of coordination for lolitrem B intoxication. Lolitrem B intoxication was demonstrated to cause a marked and dose dependent decline in latency until falling with peak effect at around two hours and duration of effect around 48 hours (Imlach et al. 2008).

The parallel rod test is a relatively recently developed test that has the advantage of being able to test both locomotion (via video tracking) and gait ataxia (by measuring foot slips) (Kamens et al. 2005; Kamens and Crabbe 2007). The parallel rod apparatus was designed principally for use with ethanol intoxication models of ataxia, however use with models of cerebellar ataxia have been proposed and used in limited studies (Nag et al. 2013; Rinaldi et al. 2013).
2.7.5.4 Exploratory Behaviours and Spatial Memory

Animals with naturally occurring PRGT demonstrate a number of behaviours that suggest cognitive deficits in normal exploratory behaviours either due to dysfunction of spatial memory or sensory function or both. Behavioural changes include the tendency for death or injury by misadventure, mass drowning events, erratic behaviour in yards and lack of shade seeking behaviour (Galey et al. 1991; Cunningham et al. 1993; Johnstone 2010; Reed et al. 2011a; Combs et al. 2014). Neurological changes suggesting sensory dysfunction include strabismus, nystagmus, hyperaesthesia, allodynia and ataxia (Johnstone 2010; Combs et al. 2014). Despite this very little attention has been paid to the role of dysfunction in normal exploratory behaviours in the aetiology of PRGT.

2.7.5.5 Barnes Maze

Spatial memory is processed and stored in the hippocampus (O'Keefe and Dostrovsky 1971; Hampson et al. 1999) The classic test for spatial learning and memory is the Morris water maze, where an animal swims in a small circular pool to find a submerged platform, often initially marked out by a flag, but then later the flag is removed to test memory of spatial cues without being able to visual reference for the object itself (the platform is unseen) (Vorhees and Williams 2006). However this test would be unsuitable due to the amount of tremor the mice may display and also the Morris water maze being preferable for testing in rats rather than mice (mice not being good swimmers).

The Barnes maze is a similar test to the Morris water maze (Barnes 1979; Berta et al. 2007; O’leary and Brown 2009) in that it is a single “escape” maze. However the Barnes maze does not involve swimming but rather exploring a circular field. To mask the escape hole the Barnes maze is bound by holes around its border although only the exit hole leads to a small chamber the rodent can hide in. The Barnes maze has been demonstrated to be useful in testing spatial learning and memory in mice (Pritchett and Mulder 2003; Berta et al. 2007; O’leary and Brown 2009).

2.7.5.6 Novel Object Recognition and Novel Arena

Rodents show a preference for exploring novel objects over familiar objects (Ennaceur and Delacour 1988; Antunes and Biala 2012). The novel object recognition (NOR) test, utilises this characteristic to test for visual recognition and contextual memory (Frick and Gresack 2003, Antunes and Biala 2012).
In NOR tests animals are familiarised with a novel arena and two objects. Then during testing one object is removed and replaced with a novel object. The time a mouse spends investigating a novel object compared to an old object relies on the inquisitive behaviour and tendency of mice to investigate new objects. It does however rely on the object being of interest to the animal, factors can cause stress (such as noise, bright light, new environment) will reduce positive affective behaviours such as exploration and must be minimised (Vogel-Ciernia and Wood 2014). Also species, sex, strain and age differences have been noted, particularly with regards to preference of novelty (Ennaceur and Delacour 1988; Burke et al. 2010; Antunes and Biala 2012).

Forebrain regions including the hippocampus that are involved in long term memory are tested using this approach (Antunes and Biala 2012). With shorter intervals between tests (approx. 1 min) some have suggested it is a pure test of working memory (Ennaceur and Delacour 1988). By moving an old object spatial memory can also be investigated using this test, although others have used the Morris water maze to demonstrate situations, such as lesions in the perirhinal cortex, where animals have novel object deficits but not spatial memory deficits (Baxter 2010). This supports the concept of specialisation of memory functions, and makes the NOR test a useful comparator test to the Barnes Maze (Baxter 2010). Although context, recognition and potentially spatial memory are examined in the NOR test it is not true that time spent with one object or another can be considered an indicator of depth of memory (Antunes and Biala 2012).

2.7.5.7 Investigation of Vascular and Cardiac Changes with Lolitrem B and BK channel Blockade.

Calcium gated potassium channels (BK) play an important role in vascular tone and blood pressure with activation of BK channels hyperpolarising smooth muscle cell membranes and causing muscle relaxation, reduced vascular tone and blood pressure (Jaggar et al. 1998; Brenner et al. 2000; Grimm and Sansom 2010). The renin-angiotensin-aldosterone system is thought to play a role in regulation of BK channel activity although the mechanisms of regulation are complex (Brozovich et al. 2016; Krishnamoorthy-Natarajan and Koide 2016; Li et al. 2017; Rieg et al. 2007; Zhang et al. 2017).

Hypertensive states have been linked to BK channel dysfunction in several disease states and in BK<sup>−/−</sup> mice (Sausbier et al. 2005; Grimm et al. 2009b; Grimm and Sansom 2010;
Holtzclaw et al. 2011). Toxins that cause BK channel blockade have also been demonstrated to increase vascular smooth muscle tone causing hypertension (DeFarias et al. 1996; McLeay and Smith 1999; Imlach et al. 2010). In situations where lolitrem B and ergovaline are both present a strong synergism of effect on smooth muscle has been demonstrated and this is likely to be clinically relevant (Dalziel et al. 2013). Synergism in neurological effects has not been demonstrated but is plausible.

2.7.5.8 Exploring the Activity of Lolitre B using a Marker of Central Nervous System Activity: c-Fos

c-Fos is the protein product of the immediate early gene c-fos, it is a 380 amino acid protein with a leucine zipper region for DNA binding and is a well described marker of neuronal activity (Dragunow and Faull 1989; Bullitt 1990; Gao and Ji 2009; Szalóki et al. 2015). c-Fos plays an important regulatory role in DNA transcription and induction of c-fos occurs rapidly following exposure of cells to a range of agents, including those trigger mitogens and cell differentiation (Curran and Morgan 1987). From the late 1980’s it became clear that following afferent stimulation to the spinal cord and brain, increased c-Fos immunoreactivity would very rapidly occur in neuronal cell nuclei and as such c-Fos could be used as a marker of neuronal activity and potentially as a marker of neuronal pathways (Dragunow and Robertson 1987a; Dragunow and Robertson 1987b; Hunt et al. 1987; Dragunow and Faull 1989). Later c-Fos was demonstrated to be a good marker of pain and stress neuronal pathways (Senba et al. 1993; Harris 1998).

The expression however of c-Fos in the cerebellum appears to be somewhat unique. Stimulation of the cerebellum with harmaline induced a loss of c-Fos activity in the soma and dendrites of Purkinje neurons but increased c-Fos expression in the nuclei of molecular layer and granular layer neurons. C-Fos was not expressed in the nucleus of Purkinje cells (Tian and Bishop 2002; Handforth 2016).

2.7.6 Summary of PRGT Animal Models

Elucidating a detailed understanding of neurological changes, particularly behavioural or cognitive changes, that occur in response to pasture derived neurotoxins presenting in livestock is challenging. Although methods of assessing gait, behavioural responses, learning and memory have been established in ruminants, including sheep, they pose numerous logistical problems for measurement in the field, and are not as well established
as similar models in rodents (Edwards et al. 1996; 1997; Lee et al. 2006; Edwards et al. 2011). When undertaking studies to evaluate both the effect and mechanism of action of toxins, although species specific-studies are required, animal cost and housing requirements make working with small or large ruminants particularly difficult whereas other validated animal models exist (Morton and Howland 2013).

Rodents, by contrast, are relatively inexpensive to maintain compared to ruminants, and provide a number of well characterised experimental models known to extrapolate to other species with high level of fidelity (Miwa et al. 2006; Miwa 2007). Rodents have been used extensively in studies characterising tremorgenic toxins, including harmaline and penitrem A (Montigny and Lamarre 1975; Milner et al. 1995; Breton et al. 1998; Cavanagh et al. 1998; Martin et al. 2005; Miwa et al. 2006). Rodent models also provide a large number of well recognised testing modalities with which to characterise neurological changes due to intoxication. These include the Barnes maze (cognition/spatial learning and memory), the parallel rod (balance and coordination), piezoelectric pressure sensor (movement/tremor) and the novel object recognition (NOR) test (positive and negative bias/fear responses and conditioning/working memory/visual recognition) (Lehtinen and Gothoni 1985; May 2003; Kamens et al. 2005; Berta et al. 2007; Kamens and Crabbe 2007; Antunes and Biala 2012). These tests examine specific components of normal neurological function allowing the underlying mechanism of action of toxic compounds to be tested to a high level of sensitivity and duplication using this system.

In addition to defining tremor syndromes, rodent models have also been used extensively to investigate potential therapeutic treatments for induced or pathological tremorgenic conditions, such as Parkinson’s disease and essential tremor in humans (Milner et al. 1995; Rozas et al. 1997; Beal 2001; Dauer and Przedborski 2003; Bové et al. 2005; Martin et al. 2005; Paterson et al. 2009). For example, the tremorgenic toxin harmaline has been extensively used as a model to investigate candidate drugs for the treatment of essential tremor (Sinton et al. 1989; Tariq et al. 2001; Martin et al. 2005; Martin and Handforth 2006; Paterson et al. 2009).

To date, impacts of lolitrem B intoxication on neurological systems other than those involved in movement have not been undertaken, however lolitrem B intoxicated
livestock exhibit neurological changes in addition to movement disorder and tremor. Hyphaesthesia, disorientation and aggression have been noted in cattle and horses suffering from PRGT (Johnstone 2010; Reed et al. 2011a; Johnstone et al. 2012; Combs et al. 2014). Reed et al. (2011a) also noted that intoxicated sheep failed to use shade on hot days when compared to animals treated with a toxin binder, although the underlying reason for this difference is unclear. PRGT has also occasionally been associated with incidences of mass drownings, suggesting alterations in mentation in intoxicated animals (Cunningham et al. 1993).

Evidence from field cases (Gallagher et al. 1982b; Reed 2009; Reed et al. 2011c; Combs M 2014) suggests that some of the behavioural changes observed in animals suffering from PRGT may be related to deficits in spatial orientation, spatial learning and/or spatial memory in addition to those of movement and balance. Spatial learning and memory are thought to be primarily governed by the hippocampus, however there is growing evidence that the cerebellum also plays into this form of memory and learning (Petrosini et al. 1996; Lalonde and Strazielle 2003; Brandt et al. 2005; Passot et al. 2012).

The mechanism by which indole diterpenoid neurotoxins exert their activity in the brain are not fully understood. Paxilline and lolitrem B are both thought to create a BK channel blockade (Knaus et al. 1994; Nardi and Olesen 2008; Imlach et al. 2009; Imlach et al. 2011; Zhou and Lingle 2014) and BK channel blockade has previously been demonstrated to impair learning generally (Matthews and Disterhoft 2009) and spatial learning specifically (Mirkowski 2013; Typlt et al. 2013). Together these findings suggest a role for these toxins in impeding normal spatial learning and memory function in intoxicated animals.

Other neurological mechanisms may also play a role in behavioural changes observed in PRGT-affected animals. Harmaline induced cerebellar dysfunction and essential tremor have also been associated with increased emotional stress (Hilber and Chapillon 2005; Chandran and Pal 2012; Moreno-Rius 2018). Additionally, Reed et al. (2011a) observed that animals exposed to PRGT-toxic pastures were not visibly ataxic but clustered in close groups and failed to seek shade even in high temperatures; a behavioural model such as ‘behavioural despair’ in rodents might explain this behaviour (Porsolt et al. 1977;1978).
2.8 Potential Therapeutic Agents for Treatment of PRGT

Currently the only therapeutic thought likely to be effective for PRGT is pentobarbitone anaesthesia (Smith et al. 1997). This treatment is completely unsuitable for farm applications as it is only viable for individual animals that can be intensively monitored. Other treatment measures for PRGT have focused primarily on techniques to reduce clinical disease expression such as reducing husbandry procedures or reducing exposure by trying to move animals to other pasture varieties. Prevention has focused primarily on the use of novel endophytes pasture varieties which have a less pathogenic toxin profile. The reason therapeutic agents have not been investigated is rarely stated although a number of objections can be postulated, including costs of treatment, the lack of an apparent available agents and the relative lack of focus on the animal welfare aspects of PRGT. However, despite novel endophytes varieties being available for many years PRGT remains a major concern. The reasons for this are again complex, but include economic cost of pasture renovation and reduced longevity of novel varieties, the unsuitability of land for pasture renovation and the difficulty of identifying novel endophyte varieties that are resistant to pests but also lack a toxicity profile in grazing animals.

In such a situation a review of potential therapeutic agents, to reduce the toxic effect to animals when grazing endophyte infected pasture, to allow husbandry procedures to be undertaken and for treatment of severely affected animals in major PRGT outbreaks, would seem like a sensible next step. The following section will address aspects of the primary therapeutic candidate, which was studied in this thesis, namely bromide, followed by a brief examination of a number of other potential therapeutic agents.

2.8.1 Bromide

2.8.1.1 History of use in Human and Veterinary Medicine

Bromide was the first antiepileptic therapy available in the era of modern medicine. Sir Charles Locock first reported the use of potassium bromide as a therapy for ‘hysterical epilepsy’ in 1857 (Sieveking 1857; Joynt 1974; Ban 2001). Bland Radcliffe and Sir Samuel Wilks also made significant contributions to early medical understanding of bromide’s use, with published findings in 1860 and 1866 respectively; reporting the successful use of bromide to treat epilepsy (Radcliffe 1860; Wilks 1861; Wilks 1875).
Some consider bromide as one of the first drugs of the modern era of medicine (Friedlander 1986; 2000).

Until the advent of phenobarbitone in 1912, potassium bromide was the only antiepileptic therapy available (Korinthenberg et al. 2007). Phenobarbitone is a potent GABA agonist and inhibitor of neuronal excitation, making it an excellent anti-epileptic drug. With the advent of phenobarbitone in 1912 and later the related drug phenytoin in 1937 the use of bromide declined (Ryan and Baumann 1999; Meierkord et al. 2000; Korinthenberg et al. 2007). Bromide however continued to be widely used in over-the-counter remedies for stress, anxiety and insomnia during the mid 20th century (Everly Jr et al. 1981; Ban 2001). But, with growing understanding of its abuse, along with a growing list of nervous system, skin and gastrointestinal side effects, bromide was eventually removed from over-the-counter sale in most western countries (Friedlander 2000).

Epilepsy has been observed in rodents, cats, dogs, horses, cattle, goats, non-human primates and humans, as well as other species (Chandler 2006). Phenobarbitone and potassium bromide remain the most commonly used antiepileptic drugs in veterinary medicine, with other therapeutic options including levetiracetam, impetion, zonisamide, gabapentin, and diazepam (Podell 1998; Volk et al. 2008; Dewey et al. 2009; Podell et al. 2016). In a study of 122 dogs on bromide therapy a reduction in seizure frequency of equal or greater than 50% was achieved in 72% of cases (Trepanier et al. 1998). In another study looking at 23 refractory cases of canine epilepsy, bromide therapy resulted in 83% of patients reducing seizure frequency, however in this study bromide was used in combination with phenobarbitone, not as a sole agent (Podell and Fenner 1993).

The therapeutic range in dogs is typically sighted as 1-2mg/ml, although Podell (1998) states levels over 2.5mg/ml may be required when using bromide as a sole therapy. Trepanier et al. (1998) report that when using bromide as a sole therapy the acceptable therapeutic range is 0.9 to 3mg/ml. In contrast however, Yohn et al. (1992) sites a case of significant toxicosis with a bromide serum level of 2.7mg/ml. In reality the level at which toxicosis may be seen in dogs is variable and so serum bromide levels can only be used as a guide for toxicity (Yohn et al. 1992; Trepanier et al. 1998).

Chronic bromide intoxication in humans results in a group of clinical signs referred to as bromism (Carney 1971; McDanal et al. 1974). Clinical signs include impaired sensorium,
dementia, psychosis, dysphagia, skin eruptions and pseudohyperchloraemia (Carney 1971; Oglesby et al. 1973; McDanal et al. 1974; Trump and Hochberg 1976; Yu et al. 2004). Interestingly, there have more recently been descriptions of bromide induced ataxia being cerebellar in origin, including one report of two patients with chronic abuse who had cerebellar atrophy; these findings may highlight a particular sensitivity to bromide in this region of the brain (Van Balkom et al. 1985; Yu et al. 2004; Rossmeisl Jr and Inzana 2009).

In contrast to humans, bromism is relatively uncommon in dogs (Yohn et al. 1992; Rossmeisl Jr and Inzana 2009; Peacock and Smart 2013), probably because of the lack of tendency to abuse. Bromism in canine patients presents in a heterogeneous manner with clinical signs varying from central nervous system excitability to stupor/coma, upper or lower motor neuron para- or tetraparesis and cerebellar ataxia (Yohn et al. 1992; Rossmeisl Jr and Inzana 2009). In dogs, other side effects can include lung irritation, polyuria, polydipsia, polyphagia, and pancreatitis (Gaskill and Cribb 2000; Bagley 2005; Chang et al. 2006; Kluger et al. 2008; Rossmeisl Jr and Inzana 2009; Baird-Heinz et al. 2012). Unlike with humans, skin eruptions do not appear to occur (Rossmeisl Jr and Inzana 2009). Bromide therapy used for seizure management is typically well tolerated and side effects are usually mild, being alleviated by dosage adjustment. High initial loading doses have been associated with gastrointestinal irritation, sedation, ataxia and hindlimb paresis which can progress to quadriplegia if toxicity is not correctly identified (Sisson 1997; March et al. 2002; Rossmeisl Jr and Inzana 2009; Baird-Heinz et al. 2012).

Bromide has not been used widely in other species, although it has been used for behavioural modification in equines (Raidal and Edwards 2008). In cats bromide is not used for anti-seizure therapy due to the risk of pulmonary disease, presumably from aspiration (Bertolani et al. 2012).

2.8.2 **Bromide Mechanism of Action**

The mechanism for bromide’s antiepileptic activity has not been extensively researched, as bromide use predates most modern neurological research techniques. As such the exact mechanism of action for bromide remains unclear; although it appears to be broad and possibly multi-modal in action (Goodwin et al. 1969; Ryan and Baumann 1999;
Meierkord et al. (2000). It is known that bromide enters cells via chloride (anionic) channels, and appears to have a direct action on the cell membrane, decreasing neuronal excitability (Dowling 1994; Ryan and Baumann 1999; Meierkord et al. 2000; Chandler 2006). Chloride channels are membrane bound proteinaceous pores and all anionic electrolytes can pass through these channels (including bromide). Anionic channels vary in structure and function, including sensitivity to calcium, voltage and volume activation (Jentsch et al. 2002; Alexander et al. 2011). As such there is also significant variation in permeability of bromide, some channels having a higher bromide permeability than chloride, resulting in hyperpolarisation of the cell membrane (Alexander et al. 2011).

Another suggested mechanism for bromide activity is that bromide increases activity of the inhibitory neurotransmitter, γ-aminobutyric acid (GABA). However this GABAergic activity is disputed; one in vivo study conducted in rats identified the GABAergic inhibitory effects of bromide as not being profound (Balcar et al. 1987), while other in vitro studies have identified a distinct increase in GABAergic inhibitory effects (Goodwin et al. 1969; Suzuki et al. 1994; Meierkord et al. 2000). GABA receptors are an extremely heterogeneous group of receptors and the effects of bromide are likely to be complex (Bormann 2000; Rudolph et al. 2001). Robertson (1989) reported that GABA activated chloride channels in the dorsal root ganglion had increased permeability to bromide over chloride and also proposed that bromide may bind to GABA receptors and prolong time of GABA binding. Llano et al. (1991) reported that GABA(A) receptors coupled to calcium activated chloride channels play an important role in GABAergic regulation of Purkinje cell excitability. These findings may have significant implications for the use of bromide as a therapy for lolitrem B toxicity in that bromide’s effect on Purkinje neurons may be profound.

2.8.3 Bromide Pharmacokinetics

The pharmacokinetic properties of bromide in sheep have been investigated by Quast et al. (2015). They found that bromide is rapidly absorbed after oral administration with a high oral bioavailability of 92%. The terminal half-life ($t_{1/2}$) of bromide in this study following oral administration was 14.5 days. The $t_{1/2}$ in humans has been reported as 12 days, 15 to 69 days in dogs, 10 days in cats and 3.1 days in the horse (Söremark 1960; Bagley 1992; Trepanier and Babish 1995a; Boothe et al. 2002; March et al. 2002; Bagley 2005; Raidal and Edwards 2008). The large reported variation in $t_{1/2}$ is likely secondary
to varying levels of dietary NaCl (Trepanier and Babish 1995b; Quast et al. 2015). One unique feature of bromide pharmacokinetics in sheep is the observation of a number of secondary peaks in serum bromide concentration that suggests gastrointestinal cyclic redistribution, perhaps via cycling through the saliva, as chloride (and therefore bromide) secretion drives salivary production (Burgen 1963; Beal 1980; Melvin 1999; Quast et al. 2015). Bromide is also concentrated within gastric secretions, supporting the hypothesis of bromide gastrointestinal recirculation (Ullberg et al. 1964).

The distribution of bromide and chloride throughout most interstitial fluid is similar, however the distribution into the cerebrospinal fluid (CSF) varies in two ways: the CSF level is lower and the rate to equilibrium is slower than in other tissues (Wallace et al. 1939b; Wallace et al. 1940a; Hunter et al. 1954; Bourdillon et al. 1957). Studies in dogs demonstrated that bromide is distributed into the CSF via the cisterna magna (Wallace et al. 1939a; Wallace et al. 1940b). Equilibrium after intravenous infusion takes seven hours, which is significantly slower than distribution into other interstitial fluids (Wallace et al. 1939b; Wallace et al. 1940a). The ratio of bromide in serum to CSF varies between studies but is approximately 3:1, although Bito et al. (1966) quote a ratio of 1.5:1. Any disease affecting the blood brain barrier can alter this ratio (Hunter et al. 1954; Bourdillon et al. 1957; Bito et al. 1966). The concentration of bromide within the CSF and brain was significantly higher than the other halides measured (excluding chloride), demonstrating the selective nature of anion channels within the blood brain barrier (Wallace et al. 1939b; Ullberg et al. 1964; Alexander et al. 2011).

Bromide, being a simple ion, undergoes no hepatic biotransformation and the excretion of bromide is almost entirely via the urine. Bromide is freely filtered at the glomerulus and reabsorption occurs within the renal tubules, the traditional view is that absorption occur in competition with chloride due to their similar electrochemical properties (Rauws and Van Logten 1975; Rauws 1983; Trepanier and Babish 1995b). However a more biologically plausible explanation was proposed by Pavelka et al. (2005) who demonstrated, using sodium salts in the absence of chloride, that retention or excretion of sodium is infact the major factor controlling the rate of renal bromide excretion. Where sodium levels are low sodium retention occurs and bromide is retained as a suitable anion for co-transport with sodium and likewise where sodium levels are high bromide is also
excreted alongside sodium. The point however, is somewhat theoretical as the most common source of sodium in the diet is in the form of NaCl.

2.8.4 Analysis of Serum Bromide

Colorimetric spectrophotometry is one of the simplest and most common analytic techniques for determining serum bromide concentrations (Goodwin 1971; Oglesby et al. 1973; Trepanier and Babish 1995a; Raidal and Edwards 2008; Quast et al. 2015). Other methods used include anion-exchange chromatography (Miller et al. 1989; Wong et al. 1989; Fielding et al. 2003; Ho et al. 2010), cyclic voltammetry (Arai et al. 1996), potentiometric flow injection (Katsu et al. 1997) and high performance liquid chromatography (HPLC).

2.8.5 Other Potential Therapeutic Agents

No drugs have been studied for efficacy in treating lolitrem B intoxication, but pentobarbitone and benzodiazepines have been demonstrated to be effective for other tremorgenic mycotoxins (Peterson et al. 1982; Cavanagh et al. 1998; McLeay et al. 1999). The mechanism of action of barbiturates is still contested although there is some agreement that they potentiate GABA(A) receptor activity (Ho and Harris; 1981; Löscher and Rogawski 2012). Benzodiazepines have greater consensus with regards to mechanism of action with the primary binding site being the GABA(A) receptor (Haefely 1978; Costa and Guidotti 1979; Campo-Soria et al. 2006). Both these classes of drugs have significant limitation for use in the grazing farm animals. Barbiturates in particular have a narrow window of safety and tend to cause profound sedation, recumbency with a significant risk of death (Livanainen and Savolainen 1983; Boothe 1998; Pagel and Parnes 2001; Verster et al. 2004; Tipold et al. 2015). Despite this, examples of use of barbiturates in horse and dairy cattle have been reported, although these drugs are used on an individual basis rather than at the herd level (Braund et al. 1971; Bennink et al. 1973; Ravis et al. 1987). Benzodiazepines have been used as sedatives in ruminants and also proposed as appetite stimulants, although expense, short duration of activity and risk of significant side effects would be concerns for herd level treatment of PRGT (Baile and McLaughlin 1979; Rehm and Schatzmann 1984; Baile and McLaughlin 1987; Abrahamsen 2013). Both drugs are rapidly metabolised in ruminant species increasing
cost again and making prophylactic use impractical (Swick et al. 1983; Abrahamsen 2013).

Within Australia, there are some commercially available products that claim to prevent the effects caused by endophyte alkaloids. The two products reported to have some effect are Elitox® made by Impextraco (Belgium) and Mycofix Plus® made by Biomin (Austria). While these products have not been developed specifically for perennial ryegrass endophytes they show some efficacy (Cummins 2008). Mycofix Plus® can reportedly deactivate mycotoxins either by irreversible binding to toxins or through enzymatic deactivation. A study identified lambs administered Mycofix Plus® grazing endophyte infected perennial ryegrass pastures had improved weight gain, were more inclined to seek shade in extreme temperatures and had lower respiratory rates (Reed et al. 2011a). The study concluded that an increased dosage may sustain higher concentrations in the rumen, increasing its efficacy (Reed et al. 2011a).

Ethanol has a known ability to activate BK channels (Chu and Treistman 1997; Bukiya et al. 2014), it also has GABA-ergic activity (Sundstrom-Poromaa et al. 2002; Koob 2004; Hanchar et al. 2005). Ethanol is effective in treatment of cerebellar tremors induced by harmaline intoxication (Sinton et al. 1989; Handforth 2012) and has proven efficacy in treatment of clinically similar tremor in humans (Growdon et al. 1975; Koller and Biary 1984b; Koller and Biary 1984; Sadeghi 2010). Alcohol does however have a number of theoretical drawbacks when thinking about its use for the treatment of PRGT in grazing livestock. Firstly, a suitable modality of treatment is not clear; dose rates for alcohol in sheep are high and probably intravenous administration is required to avoid rumen degradation or excessive first pass hepatic metabolism (Ngai et al. 2008). Ethanol is heavily metabolized in the rumen and rapidly metabolized by the liver in ruminants (Pradhan and Hemken 1970; Kristensen et al. 2007). Additionally a well characterised side effect of tremor therapy with alcohol is the exacerbation of tremor post therapy. This is particularly well documented with the treatment of essential tremor (Growdon et al. 1975; Koller and Biary 1984; Klebe et al. 2005; Sadeghi 2010).

Both carbenoxolone and mefloquine are proposed to reduce tremor and seizure activity by reducing synchronicity of neuronal firing of neuronal networks. In the case of cerebellar tremor, the site of action is assumed to be in the inferior olive or the cerebellum.
Reduction of synchronous neuronal firing is thought to occur via gap junction blockade (Rozental et al. 2001; Cruikshank et al. 2004; Martin and Handforth 2006), although other authors have disputed this and suggested GABAergic actions may be responsible for the neuronal activity of these drugs (Rouach et al. 2003; Connors 2012). Despite some controversy over mode of action both carbenoxolone and mefloquine have demonstrated efficacy as drugs for the treatment of harmanline-induced cerebellar tremor (Martin et al. 2005; Martin and Handforth 2006). Carbenoxolone, because of its multitude of effects on electrolytes, mineralo- and glucocorticoid activity and because of the unknown pharmacology in sheep, should be considered with caution as a large animal therapy (Turpie and Thomson 1965; Davies et al. 1974; Stewart et al. 1990; Sandeep et al. 2005).

Propranolol, like carbenoxolone and mefloquine has been demonstrated to be effective in the treatment of harmaline induced tremor and is used extensively in the treatment of essential tremor in humans (Dupont et al. 1973; Koller and Biary 1984; Zesiewicz et al. 2002; Martin et al. 2005). The mechanism of action for propranolol is via beta-adrenergic blockade, and the site of action may be principally peripheral rather than central (Young et al. 1975; Jefferson et al. 1979; Koller and Biary 1984; Ondo 2016). The use of beta-adrenergic antagonists that are more water soluble and therefore less centrally acting (such as sotalol) have been shown to be equally effective to propranolol and generate less sedation, which may improve their clinical usefulness in grazing animals (Ondo 2016). However expense and duration of activity remain a concern for this drug.

2.8.6 Other Management Strategies

The clinical signs exhibited by tremorgenic mycotoxins are reversible without therapeutic intervention if they are mild. This is reliant on animals being removed from endophyte infected pastures (Gupta 2007). Severely affected animals, particularly those that cannot stand need significant supportive care to survive including hand provision of water, feed and insuring that animals do not remain in lateral recumbency, often by placing them in a trench to support them (Rendell 2016). In extensive systems, grazing management and stocking densities play a vital role in preventing toxicosis. Stock should have access to young growing plants in pastures not dominated by perennial ryegrass, particularly in the later summer to autumn months (Gupta 2007). However, this is not always possible in perennial ryegrass dominant regions.
2.9 HYPOTHESES AND RESEARCH OBJECTIVES

Perennial ryegrass toxicosis (PRGT) is a major disease in grazing animals in southern Australia and New Zealand with significant impacts on farm productivity and animal welfare. Historically research has focused primarily on identifying the source of the disease, the toxins involved and understanding the impacts on productivity. Relatively little research has been conducted into the neurological changes observed in affected livestock and no research looking directly at therapy has been conducted.

The focus of the investigations reported in this thesis is to determine if bromide might be a useful on-farm therapeutic. However, before these questions could be addressed a better understanding of the neurological disease was needed and improved means of measuring those neurological signs would be required if evidence of therapeutic effect were to be established.

The hypotheses for the studies presented are:

- Ovine and murine disease models can be developed that improve characterisation of tremor state and greatly increase overall knowledge regarding the neurological dysfunction caused by PRGT;
- The developed PRGT models can contain objective measures of neurological disease suitable for assessing efficacy of a therapeutic agent;
- Bromide, a primary candidate therapeutic for PRGT, can be assessed for efficacy using objective measures in both murine and ovine model of PRGT.

In order to interrogate these hypotheses, the following research objectives were established:

- To investigate naturally occurring outbreaks of PRGT to inform development of laboratory models by assessing neurological, clinicopathological and neuropathological changes;
- Development of a murine PRGT disease model to improve understanding of neurological changes and better characterise tremor;
• Development of an ovine PRGT model, using the tremorgenic neurotoxin lolitrem B, to improve understanding of distinct neurological changes associated with this condition and better characterise tremor in affected animals;

• Using quantitatively verified tools in a murine disease model to assess the efficacy of bromide for treating the neurological effects of lolitrem B intoxication;

• And finally, use quantitatively verifiable testing within an ovine PRGT model to validate bromide as a potential on-farm therapeutic for PRGT in sheep.
Chapter 3 CLINICAL PRESENTATION OF PERENNIAL RYEGRASS TOXICOSIS OUTBREAK IN AUSTRALIA.

This is the first of the research chapters in this thesis. The aim of this chapter is to improve the understanding of disease aetiology and clinical expression by studying a naturally occurring outbreak of PRGT. This chapter is based on clinical data collected during the 2011 outbreak of PRGT in south-western Victoria. The study forms an important part of this thesis as clinical findings, including presentation of neurological signs and the presence of dehydration form important parts of later studies, particularly the two later chapters where water intake, dehydration and gait analysis form important parts of laboratory trials of a PRGT model (Chapter 6 & 7). The observation and classification of Type 1/ Type 2 gait changes in this study form the basis of later analysis (Chapter 7) where set clinical criteria, based on gait changes, are used to define a time point at which therapeutic intervention is to take place.

This study was the first time significant clinicopathological data was collected from field cases of PRGT. Also by collating pasture, faecal and tissue lolitrem B levels with histopathological data this paper removes any controversy surrounding the histopathological diagnosis of PRGT.

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Evidence of dehydration and electrolyte disturbances in cases of perennial ryegrass toxicosis in Australian sheep

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ABSTRACT

Case report: Perennial ryegrass toxicosis (PRGT) is a common disease entity in Australia, presenting as an association of clinical signs including alterations in normal behavioural, ataxia (‘staggers’), ill thrift and gastrointestinal dysfunction (‘scours’). Clinical signs can range in severity from mild (gait abnormalities and failure to thrive) to severe (seizures, lateral recumbency and death). Presentation across the flock is usually highly variable. PRGT is caused by toxins produced by the endophytic fungus Neotyphodium lolii, a symbiont of perennial ryegrass that is present in pastures across the temperate regions of Australia and Tasmania. A particular feature of PRGT in Australia is the occasional occurrence of large-scale sheep losses, suggesting other factors are influencing mortality rates compared with other PRGT risk zones such as North America and New Zealand.
During 2011, producers in the state of Victoria experienced a mild outbreak of PRGT that affected large numbers of animals but with limited mortalities. Clinical samples taken from affected sheep showed a high incidence of dehydration and electrolyte abnormalities.

Conclusion: We speculate that changes in hydration status may be a contributory aetiological factor in those years in which high numbers of deaths are associated with PRGT outbreaks in Australia.

**Keywords** dehydration; ergovaline; lolitrem B; perennial ryegrass; sheep; staggers; toxicosis

**Abbreviations**: DM, dry matter; HPLC, high-performance liquid chromatography; MCHC, mean cell haemoglobin concentration; PRGT, perennial ryegrass toxicosis; pTP, plasma total protein

### 3.1 Introduction

Perennial ryegrass toxicosis (PRGT) is a toxicity syndrome affecting livestock grazing on perennial ryegrass infected with the wild-type endophytic fungi, *Neotyphodium lolii*, which produces the potent indole diterpenoid toxin, lolitrem B, and the ergot alkaloid ergovaline (Gallagher *et al.* 1982b; Cheeke 1995). Clinical signs of PRGT include abnormal behaviour, ataxia (‘staggers’), ill thrift and gastrointestinal dysfunction (‘scours’) (Cunningham and Hartley 1959). Clinical signs range in severity from mild gait abnormalities and failure to thrive, to severe seizures, lateral recumbency and death (Finnie *et al.* 2011).

Mild outbreaks result mainly in subclinical production losses and management and animal welfare issues for affected producers whereas severe outbreaks can involve significant stock losses. Clinical presentation is usually highly variable, with season, breed, sex, age and production status all affecting the severity of clinical signs in the flock or herd (Finnie *et al.* 2011; Reed *et al.* 2011c). A particular feature of PRGT in Australia is the occasional occurrence of large-scale sheep losses, suggesting other factors are
influencing mortality rates compared with other PRGT risk zones such as North America and New Zealand.

Despite the prevalence of PRGT in Australia, there is little published data on the clinicopathological changes found in animals affected in the field. In this study, we report findings from PRGT cases presenting during the peak of a mild PRGT outbreak in Victoria, Australia, in 2011. Pasture samples were collected from eight properties in the locality, as well as pathological samples from 13 sheep with clinical signs consistent with PRGT and ranging from ambulatory but ataxic through to prolonged recumbency and/or severe and continuing neurological signs such that euthanasia was required. Neurological observations identified two key gait abnormalities associated with intoxication and clinical samples taken from affected sheep showed changes indicative of dehydration and electrolyte abnormalities. We speculate that changes in hydration status may be a contributory aetiological factor in the years when high mortality rates, and high ambient temperatures, are associated with severe PRGT outbreaks in Australia.

3.2 CASE SERIES

3.2.1 History

Clinical investigations were undertaken in March and April of 2011 during an outbreak of PRGT in the Hamilton region. Seven properties in the region were visited during this period after reports of suspected cases of PRGT (Appendix 10.1.1). Pasture samples from the properties (Appendix 10.1.2), as well as blood, faeces, brain, cerebrospinal fluid, spinal cord and adipose tissue samples, were collected from 13 sheep with clinical signs ranging from ambulatory but ataxic through to prolonged recumbency and/or severe and continuing neurological signs with poor prognosis.

All farms visited had a long history of outbreaks of PRGT prior to and including the 2011 season. Producers first noted signs 3–7 weeks prior to the veterinarian’s visit, with all reporting a generally improving trend at the time of sampling. Onset of clinical signs varied, with some noting problems in conjunction with animal husbandry procedures, such as drenching or shearing, and others noting problems when moving animals between pastures or while observing flocks or herds without intervention.
Producer estimates of the percentage of animals affected ranged from 10% to 40%. Observable cases at the time of sampling ranged from 20% to 80%, with the majority exhibiting mild motor deficits and a minority of animals in sternal or lateral recumbency. Gross losses on the farms sampled ranged from 2 animals (total flock of 2300) to 58 (total flock of 1450). In the latter, 43 animals were found drowned in a dam, with a further 15 from the same mob dying post recumbency (when they were moved from the severely toxic pasture to one known to be less toxic). During this outbreak, ambient temperatures were low and abundant green feed was available to all animals, which is not typical of outbreaks of PRGT in the Australian setting. Although this was not a severe PRGT outbreak, it still represents significant financial importance to the producers on the more severely affected properties.

3.2.2 Inclusion Criteria

In order to exclude similar cases resulting from ingestion of other tremorgenic pasture species, such as phalaris ‘staggers’, we determined six new diagnostic criteria for inclusion in the case series. (1) Severe neurological signs consistent with PRGT, including tremor, gait abnormalities, increased severity of incoordination on movement or stress, seizures, initial or continued sternal recumbency. Abnormalities of movement were determined within two distinct presentations. In type 1, animals exhibited dysfunction in initiation of movement, with a failure to correctly extend limbs in sequence while still maintaining ambulatory status. In type 2, animals exhibited a shortened, bunny-hopping gait with rigid limb hyperextension, typically falling into lateral recumbency on attempts at rapid movement. (2) Previous history of PRGT on the property and current reporting of PRGT signs by the producer. (3) Prolonged exposure to pastures containing perennial ryegrass at the time of first appearance of clinical signs. (4) Greater than 10% of the flock with current or reported gait abnormalities in the past 10 days. (5) Absence of clinical or neurological signs indicating any other disease aetiology. (6) When appropriate, histopathology and/or postmortem findings consistent with a diagnosis of PRGT.

3.2.3 Animals

Based on our criteria, 13 sheep from 7 properties in the Hamilton region were included in this study. Most animals on the properties visited showed mild to no neurological signs
at the time of examination and therefore were not suitable for selection. The animals meeting the inclusion criteria were 9 ewes, 2 wethers and 2 rams, ranging in age from 7 months to 4 years. These included composite (4), first-cross Merino (4) and Merino (4) animals and 1 Suffolk ram. Of these 13 animals, 10 were euthanased on the farm because their condition was determined on examination to be non-recoverable and a postmortem was carried out in field (thereby meeting the 6th criteria). Visual examples of cases can be seen in Appendix 10.1.4.

Three animals were not euthanased and therefore did not meet all 6 criteria, but complied with criteria 1–5. Blood samples were collected from all three and faecal samples collected from one of these cases for toxin analysis (animal E11, Table 3-1).
<table>
<thead>
<tr>
<th>Farm</th>
<th>Animal&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HCT (L/L)</th>
<th>Na (mmol/L)</th>
<th>Cl (mmol/L)</th>
<th>K (mmol/L)</th>
<th>Alb (g/L)</th>
<th>Urea (mmol/L)</th>
<th>Creat (µmol/L)</th>
<th>CK (U/L)</th>
<th>AST (U/L)</th>
<th>Fibrinogen (g/L)</th>
<th>pTP:fibrinogen ratio</th>
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<td>37.5</td>
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<td>845</td>
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<td>108.1</td>
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<td>38</td>
<td>9.4</td>
<td>139</td>
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<td>G 13</td>
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<td>N/T</td>
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<tr>
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<td>132–152</td>
<td>95–111</td>
<td>3.9–5.4</td>
<td>24–30</td>
<td>2.9–7.1</td>
<td>79–177</td>
<td>0–200</td>
<td>0–150</td>
<td>1.0–5.0</td>
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</table>

Values outside the normal range are in bold. <sup>a</sup>: All animals exhibited clinical and histopathological signs consistent with PRGT and all except B3, E11 and G13 were euthanased because of the severity of neurological signs. Sodium (Na), chloride (Cl), albumin (Alb) and urea concentrations for animals 1 and 10 suggest acute renal failure. AST, aspartate aminotransferase; CK, creatine kinase; Creat, creatinine; Fib,fibrinogen; HCT, haematocrit; K, potassium; NT, not tested; pTP, plasma total protein.
Values outside the normal range are in bold. All animals exhibited clinical and histopathological signs consistent with PRGT and all except B3, E11 and G13 were euthanased because of the severity of neurological signs. Sodium (Na), chloride (Cl), albumin (Alb) and urea concentrations for animals 1 and 10 suggest acute renal failure. All animals were down at presentation, and euthanased because of the severity of their neurological signs, except animals 3, 11 and 13, which were ambulatory. AST, aspartate aminotransferase; CK, creatine kinase; Creat, creatinine; Fib, fibrinogen; HCT, haematocrit; K, potassium; NT, not tested; pTP, plasma total protein.

3.2.4 Sample collection

Venous blood samples were collected in EDTA and/or plain vacutainers from all 13 animals and stored and transported on ice to maintain sample integrity prior to processing. Transport took less than 24 h. If samples needed to be stored for longer than 24 h prior to processing, plasma was centrifuged and collected and stored on ice. Haematology results are not presented for those cases. Sample analysis was performed at the Veterinary Diagnostic Laboratory, Charles Sturt University, Wagga Wagga, New South Wales. Serum biochemical screening was performed using a Konelab 30™ biochemical analyser (Thermo Fisher Scientific Australia, Scoresby, VIC, Australia). A complete blood count was performed on 10 samples using the Cell-Dyn™ 3700 system (Abbott Diagnostics, North Ryde, NSW, Australia). Blood, faeces, brain, cerebrospinal fluid, spinal cord and adipose tissue samples were collected from all the postmortem cases. Brain and spinal cord samples were processed from all the euthanased animals for routine histopathology.

Pasture samples were taken using a standard random sampling method (Reed et al. 2000) from paddocks in which affected sheep had been grazing at the time gait abnormalities were first noted by the producer. Pasture samples comprised the entire aboveground portion of the plant, including the crown, which were freeze-dried and ground prior to analysis by high-performance liquid chromatography (HPLC) for lolitrem B and ergovaline quantification (AgResearch Ltd, New Zealand) as per published protocols (Toragbidye et al. 1994; Porter 1995; Miyazaki et al. 2001).

Adipose tissue, serum and faecal and samples were collected from animals C4, D6 and D10, with faecal samples only collected from ambulatory animal E11, for toxicological
analysis by HPLC for quantification of lolitrem B10 (Southern Scientific Services Pty Ltd).

3.2.5 Neurological Signs

All recumbent animals examined had a fine tremor of the facial muscle, neck and limbs, with effects being more prominent in fore-limbs than in hindlimbs. Lateral recumbency reduced but did not completely ablate this tremor, which still could be identified on careful examination. The fine tremor would progress to a coarse tremor with attempts at movement. In the most severe cases, the tremor resembled seizures, with limb tremor, limb extension and opisthotonos. There was, however, no loss of consciousness or evidence of post-ictal neurological signs that would identify the episodes as cerebellar seizures.

Spinal reflexes followed no discernible trend with normal, mild hypo- or hyperreflexia all being noted. Sensation and withdrawal responses appeared normal in all cases. Conscious proprioceptive testing undertaken in one ambulatory animal (E11) was found to be normal despite significant tremor. Some (5/10) recumbent animals exhibited ventral strabismus, which was exacerbated by extension of the neck; otherwise, cranial nerve function appeared normal. The majority of the recumbent animals examined would take food if offered, suggesting no severe cognitive deficit in these cases and no obvious damage to the cranial nerves involved in prehension, mastication and swallowing. On one property, a number of sheep in sternal recumbency were observed to have crawled to graze despite being unable to rise independently or remain standing if assisted to do so. Animals that had been recumbent for longer periods of time (estimated to be ≥3 days) were observed to be dehydrated and showed evidence of muscle wasting and anorexia.

Large numbers of ambulatory, mildly affected animals were observed on all properties visited. These animals were observed to exhibit two distinct types of gait abnormality, which we classified as type 1 or 2 ataxia and which have not been reported in detail previously. The signs of type 1 were more pronounced as the rate of movement increased and often ended with sudden collapse on either the fore-limbs or hindlimbs, usually into sternal recumbency. Animals with mild type 1 gait abnormalities were occasionally observed to be unable to follow a desired direction of motion, such that an individual
separated from the flock would direct themselves towards the flock but would then follow an oblique path, resulting in the animal failing to reach its goal. These animals exhibited mild head and neck tremor on careful observation. A rapid eye tremor (i.e. not a true nystagmus) was noted in one animal on close observation.

Animals exhibiting type 2 ataxia typically fell into lateral recumbency on attempts at rapid movement. Initially after falling, the limbs would be rigidly extended before relaxing in lateral recumbency. Head and neck movements were usually normal, with no extension of the neck, suggesting the myoclonus was in locomotor muscle groups only and not generalised in these cases. Animals with either type of ataxia could regain a standing position after a short period of recumbency.

3.2.6 Pathological Analysis

Ten animals were euthanased and necropsy revealed no gross abnormalities of thoracic and abdominal cavities other than loss of omental fat and mild fatty changes in the liver, which are typically associated with anorexia.

In 9 of the 10 cases examined postmortem, the cerebellar histopathology comprised loss of Purkinje neurons and axonal swellings in the granule cell layer (Figure 3-1, Appendix 10.1.5). In two cases, small areas of haemorrhage were also observed within the cerebellum, although this is likely to be a non-specific finding.
Figure 3-1: Histological section (H&E) of the cerebellum from a sheep affected by perennial ryegrass toxicosis (PRGT). Histological changes that are characteristic of PRGT include axonal swelling of Purkinje neurons (P) within the granule cell layer (*). Scale bar = 100 μm.

Mean serum albumin (± SE) was 34.6 g/L (± 1.2) (normal range 24–30 g/L) (Table 3-1). Electrolyte disturbances were present in 7 of 12 animals examined, including hyperkalaemia and hypernatraemia (Table 3-1). Only one animal showed haematocrit values outside the normal range (A1; Table 3-1).

In this case series, 3 animals showed evidence of mild to moderate azotaemia (animals A1, D6, D10; Table 3-1). Of the 9 animals tested, 6 showed moderate to severe elevations in fibrinogen (Table 3-1), with the plasma total protein (pTP) to fibrinogen ratio indicating hyperfibrinogenaemia in 5/9 cases tested (defined as pTP : fibrinogen <10;11 Table 3-1). All animals showed plasma concentrations of creatine kinase (CK) above the normal range (Table 3-1). Consistent changes in any of the other biochemical parameters analysed were not noted; these included creatinine, bilirubin, alkaline phosphatase, aspartate aminotransferase, glutamate dehydrogenase, gamma-glutamyl transpeptidase,
cholesterol, glucose, bicarbonate, calcium, phosphorus, magnesium and β-hydroxybutyrate.

3.2.7 Toxin Quantification in Plant and Animal Samples

All the affected pastures inspected in this study were dominated by perennial ryegrass. In the majority of properties visited, affected animals had been removed from their original paddocks when the samples were taken. Toxin concentration ranges in the pasture samples were 0.8–3.9 mg/kg dry matter (DM) of lolitrem B and 0.2–0.8 mg/kg DM of ergovaline (Figure 3-2).

Concentrations of lolitrem B toxin in faecal samples were 1.69 mg/kg DM (animal E11), 0.21 mg/kg (animal D6) and 0.23 mg/kg (animal D4). Lolitrem B was not detected in any serum samples analysed and was only detected in white adipose tissue samples from animal D4 (0.02 mg/kg DM) and animal D10 (0.04 mg/kg DM).
Figure 3-2: Lolitrem B and ergovaline concentrations from replicate samples of perennial ryegrass from affected paddocks on seven farms (A–G) where grazing livestock exhibited clinical signs of perennial ryegrass toxicosis during autumn 2011. Pasture toxin concentrations from the published literature at which clinical signs are reported to be first observed (represented by red and blue lines for lolitrem B and ergovaline, respectively) (Porter 1995; Finch et al. 2012). DM, dry matter. For full analysis see Appendix 10.1.3.

3.3 DISCUSSION

To our knowledge, this study is the first clinical case series evaluating clinical pathological parameters from affected animals in an on-farm setting, examining clinical pathology from both surviving and non-surviving cases. The sporadic and unpredictable nature of PRGT out-breaks in Australia, coupled with the difficulty of accessing suitable cases on commercial properties, has meant that, to date, evaluation of the effects of PRGT on production livestock has been mainly achieved retrospectively. Past studies have accessed postmortem reports from government laboratories (Russell 1975; Munday et al. 1985; Cunningham et al. 1993) or used in-field trials in which very few or no animals were allowed to reach the severity of clinical signs associated with non-recovery;
therefore; postmortem sampling has been difficult to achieve (Toragbidye et al. 1994; Reed et al. 2010; Reed et al. 2011a; Finch et al. 2012).

A specific diagnosis of PRGT has historically been considered to be one of exclusion. Histopathological findings are difficult to interpret in isolation, as the diagnostic value of the classical PRGT lesion, namely, proximal axonal bodies of Purkinje cells in the cerebellum, (Parton KB 2006) is questionable. Purkinje cell loss or damage and proximal axonal swelling alone is not pathognomonic of PRGT, as these can occasionally be observed in normal animals or animals affected by other neurological disease 19 and their role in the pathogenesis of PRGT is still unclear. Therefore, a definitive diagnosis of PRGT is made by the absence of histopathological features suggestive of another neurological diagnosis and in conjunction with clinical and historical signs.

Specific toxicological testing of pasture samples is expensive and therefore has been infrequently used for field-based cases. The use of the Keogh tremor scale, (a widely used severity scale giving a score from 1 to 5) is also problematic because it is a subjective rather than objective measure of tremor intensity and involves driving the animals to assess the degree of tremor (Keogh 1973). Such comparative measures can often be difficult to apply to livestock in the field, particularly in a disease where tremor will increase both with intention and with stressors.

We defined a new set of diagnostic criteria for PRGT in this report. A key element of these criteria is a new definition of the movement disorder associated with PRGT. Animals examined in this study did not exhibit a true hypermetria, as the classic exaggeration of both flexion and extension of limbs was not present. Further, there was no increase in the degree of tremor towards the termination of a movement, another defining characteristic of cerebellar ataxia. Rather, for type 1 cases, dysdiadochokinesis, or difficulty in performing rapid sequential limb movements, would appear to be a more accurate description. In type 2 cases, for which hyperextension of limbs was a key feature, rhythmic myoclonus may be a better description of the gait abnormality. Together, these observations support a central ataxia rather than a purely cerebellar lesion.

There are a number of neurological syndromes in humans that resemble the movement disorders observed in PRGT-affected livestock. These include essential tremor or
opsoclonus–myoclonus syndrome (Sahu and Prasad 2011). Although these disorders do have a cerebellar component, they are also thought to affect associated brainstem and thalamic nuclei (Caviness et al. 1995; Hellwig et al. 2003). An improved understanding of the neurological lesion involved in PRGT may open a number of therapeutic options for prevention or management of animals in severe outbreaks.

The transition from type 1 to type 2 neurological signs may represent a progression in the severity of intoxication. Elevation in creatine kinase levels and hyperfibrinogenaemia could be also used as indicators of the severity of intoxication whereby increasing severity of tremor results in muscle trauma either from increased muscle activity because of the tremor or trauma resulting from falling. Muscle damage in PRGT cases has previously been reported in other species (Reed et al. 2010), with histopathological changes in muscle being well recognised. However, currently there are not enough data to determine whether the different types of presentation represent different degrees of intoxication, different stages of the disease process or age/sex/breed-associated differences in presentation. Collection of more data, particularly if neurological changes could be related to lolitrem B concentrations in the animal, would be potentially valuable for better classification of the severity of the disease within the flock.

3.3.1 Effects of Dehydration in PRGT Cases

The cause of the severity of clinical presentation and death in PRGT cases in Australia is poorly understood. Neurological dysfunction and gait abnormalities alone are not sufficient to result in death in the absence of other compounding factors. The 2011 PRGT outbreak in Victoria reflected an unusual season in which perennial ryegrass continued to grow throughout summer, making it more typical of the New Zealand experience, with high levels of morbidity but low mortality rates (Gallagher et al. 1982b). However, despite low ambient temperatures and abundance of feed, we found clear evidence of dehydration and electrolyte disturbances in the animals tested in this study. This observation supports a previously unreported complication of PRGT of significance to the Australian setting where ambient temperatures are generally high, an effect that will exacerbate clinical signs of PRGT in on-farm cases.
This is the first time evidence of dehydration has been reported in animals clinically affected by PRGT. Although dehydration and electrolyte imbalance might have been expected in the most severely affected (moribund) animals in this study, observation of similar changes in ambulatory animals (animals with access to plentiful green feed and water) suggests dehydration may be a contributing factor and an underlying aetiology in the pathogenesis of PRGT in Australia.

Dehydration and electrolyte imbalance is a particularly pertinent finding in the Australian setting where reduced access to green feed and higher ambient temperature have been identified as risk factors in severe outbreaks of PRGT (Reed et al. 2011c). Our clinical findings suggest that physiological stress caused by dehydration, altered gastrointestinal function and reduced mobility because of PRGT, exacerbated by environmental factors, will significantly reduce an affected animal’s ability to maintain electrolyte homeostasis. This is supported by observations in New Zealand that during drought conditions there is an increased risk of dehydration in stock; although there has to date not been clinicopathological analysis to further investigate this (Morris et al. 1995). It has been previously reported that in severe PRGT outbreaks in the Hamilton region, Victoria, average ambient temperatures were higher than normal and autumn rainfall was reduced. Despite these climatic changes, lolitrem B toxin levels in pasture were not found to be significantly different in comparison with those years in which mild outbreaks occurred (Reed et al. 2011b), suggesting other factors are involved in the variable clinical presentation of PRGT in Australia.

Of the 13 sheep, 12 showed evidence of dehydration as defined by increased concentrations of serum albumin11 above normal ranges (Table 3-1). Although elevation of albumin levels in the majority of animals examined in our study was generally not severe, the trend is significant. Dehydration in our cohort was not related to ability to freely access water as the three ambulatory animals included in this study also exhibited albumin levels considerably higher than normal range (B3, E11, G13; Table 3-1). A previous investigation of albumin levels in sheep in the Hamilton region reported levels of 27–29 g/L, although in that study the subjects were parasite-free sheep on a high plane of nutrition (Hucker and Young 1986). The same study showed albumin levels to be lower in sheep with intestinal parasites (Hucker and Young 1986). Farms affected by PRGT
often have interrupted drenching routines and this was the case on most of the farms in the present study. In such circumstances, a normal to high parasite load generally results in a lowered albumin range within the flock (Hucker and Young 1986). Haematocrit proved to be less useful as an indicator of dehydration in the animals reported in our study and our data support previous studies investigating mild to moderate dehydration in sheep, in which haematocrit was observed to be within normal limits in sheep experiencing water restriction while the plasma albumin level was routinely elevated (Igbokwe 1993; Casamassima et al. 2008).

Hyperkalaemia and hypernatraemia both have the potential to induce muscle weakness, hyperreflexia and ataxia, thereby exacerbating the clinical signs of PRGT. As the mean cell haemoglobin concentrations (MCHC) were within or very close to the normal range (defined as 310–340 g/L) in animals with hyperkalaemia (animal A1: K, 8.8 mmol/L; MCHC, 306 g/L; animal A2, K, 6.5 mmol/L; MCHC, 325 g/L; animal B3, K, 5.8 mmol/L; MCHC, 331 g/L), as well as those with normal potassium concentrations, this suggests that the increased levels of serum potassium observed were not an artefact of sample processing, transport conditions or intravascular haemolysis.

Three animals in our cohort showed evidence of mild to moderate azotaemia (A1, D6, D10; Table 3-1), which may be related to hydration status, indicative of muscle damage or some other unidentified renal pathology.

Elevated fibrinogen can be indicative of both inflammation and/or haemoconcentration and both conditions may have affected the pTP: fibrinogen ratios in our cohort, with pTP : fibrinogen <10 being indicative of inflammatory changes (Stockman and Scott 2008).

Lolitrem B, the indole diterpenoid toxin produced by N. lolii, is the causal agent of the movement disorders associated with PRGT (Gallagher et al. 1981; Gallagher et al. 1982a). Lolitrem B antagonises the calcium-activated large-conductance potassium channels (BK channels), blocking their function and causing neuronal hyper-excitability in the central nervous system (Dalziel et al. 2005; Imlach et al. 2011). In the kidneys and colon, BK channels play a role in excretion of potassium, particularly when levels are elevated (Holtzclaw et al. 2011).
Multiple toxicity is a significant compounding factor in the presentation of PRGT, as the endophyte that produces lolitrem B also produces a range of other toxic substances, including the ergot alkaloid ergovaline, which previously has been speculated to be a compounding factor in PRGT cases. Ergovaline may also play an important contributory role in exacerbating the dehydration in PRGT, as it has been shown to cause heat stress in a number of species, including ruminants (Gadberry et al. 2003). Ergovaline is a dopamine D2 agonist that inhibits angiotensin II-mediated release of aldosterone, affecting its potassium excretion and water-sparing role in the kidney (Whitfield et al. 1980; Dyer 1993; Larson et al. 1995; 1999). Ergovaline also causes peripheral vasoconstriction, which, in high ambient temperatures, leads to hyperthermia and increased water loss from the respiratory tract (Gooneratne et al. 2011). Lolitrem B and ergovaline are also likely to synergistically inhibit the excretion of adrenocorticotropic hormone, thereby reducing cortisol levels and disrupting normal gastrointestinal function (Brunton et al. 2007; Johnstone et al. 2012). How this combined toxicity exacerbates the clinical signs of PRGT in on-farm conditions is hard to determine but warrants further investigation.

It has been previously reported that levels of 1.8–5 mg/kg DM of lolitrem B and 0.5–0.8 mg/kg of ergovaline in forage are the approximated threshold values for clinical signs to be observed in production animal species. However, not all samples collected from paddocks presenting clinical cases in this study reached the critical threshold for lolitrem B (only paddocks A, B D2 and F2) and the concentrations of ergovaline were all below threshold in this outbreak (Figure 3-2). There are several possible reasons for this; the entire plant (green and dry fractions, leaf and stem) was collected and analysed, but the green fraction of grass is characterised by a higher concentration of toxins and in some of the paddocks sampled it had been extensively grazed. Also, although many animals had initially gone down during a period of hot weather, they were examined in cooler wet conditions when clinical signs within the herd were improving and this may also have been reflected by the declining toxin levels in the plants.

Another possibility is that a lower level of toxin initiates clinical signs in naturally occurring outbreaks than has been previously determined. Our preliminary data suggests that a threshold of 1.5 mg/kg DM of lolitrem B may be more appropriate as an indicator
of potential clinical toxicity in pastures presenting clinical signs in ovine cases of PRGT. Although the metabolism of lolitrem B is not well understood, future studies should address the question of whether concentrations >2.0 mg/kg in faecal samples from affected animals could be used as an indicator of toxin exposure in clinical cases of PRGT and could be a useful measure of exposure at postmortem examination for PRGT diagnosis. Indeed, the concentration of lolitrem B toxin in the faecal sample from the ambulatory animal in the present study was 1.69 mg/kg DM, suggesting that this animal was still ingesting toxic pasture and that it was also sufficient to induce clinical signs. The lower toxin levels in the recumbent sheep may likewise reflect deceasing oral intake. These data suggest that the concentration of lolitrem B in faecal samples could be a useful indicator of toxicity status in both pre- and postmortem cases of PRGT.

In conclusion, the data reported in this study suggest that other factors, such as dehydration, are contributing to the high mortality observed by producers in severe PRGT outbreaks in the Australian setting. Therefore, hydration status should be considered within any management approach taken by producers and veterinarians when recommending treatment strategies for PRGT-affected livestock.

3.3.2 Acknowledgements

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Additional material relating to this chapter can be found in Appendix 10.1
Chapter 4  CHARACTERISATION OF NEUROLOGICAL DYSFUNCTION INDUCED BY THE BK CHANNEL ANTAGONISTS PAXILLINE AND LOLITREM B USING A MOUSE MODEL.

This chapter is one of two chapters in this thesis based on rodent models of PRGT. Both chapters examine the neurological effects of the lolitre B component of PRGT. The chapter has two aims, to improve the understanding of the neurological effects of lolitre B and to build a murine PRGT disease model platform for future therapeutic testing.

This chapter asks three key questions, can lolitre B induced tremor be better characterised with an objective tremor measurement? Secondly, what are the nature of lolitre B induce coordination defects? The third question comes from clinical observations in Chapter 3, as well as literature reports of behavioural changes (hyperaesthesia, alldynia, aggression, mass drowning events) that imply cognitive deficits often with a spatial element. Therefore in this chapter the cognitive effects of lolitre B were examined using novel object recognition (visual recognition and contextual memory) and the Barnes maze (spatial learning and memory). Because lolitre B is a relatively novel compound for this type of experiment a positive control was also used in the testing, the well characterised BK channel blocker paxilline.

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**A single exposure to the tremorgenic mycotoxin lolitrem B inhibits voluntary motor activity and spatial orientation but not spatial learning or memory in mice**

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**ABSTRACT**

The indole diterpenoid toxin lolitrem B is a tremorgenic agent found in the common grass species, perennial ryegrass (*Lolium perenne*). The toxin is produced by a symbiotic fungus *Epichloë festucae* (var. lolii) and ingestion of infested grass with sufficient toxin levels causes a movement disorder in grazing herbivores known as ‘ryegrass staggers’.
Beside ataxia, lolitrem B intoxicated animals frequently show indicators of cognitive dysfunction or exhibition of erratic and unpredictable behaviours during handling. Evidence from field cases in livestock and controlled feeding studies in horses have indicated that intoxication with lolitrem B may affect higher cortical or subcortical functioning. In order to define the role of lolitrem B in voluntary motor control, spatial learning and memory under controlled conditions, mice were exposed to a known dose of purified lolitrem B toxin and tremor, coordination, voluntary motor activity and spatial learning and memory assessed. Motor activity, coordination and spatial memory were compared to tremor intensity using a novel quantitative piezo-electronic tremor analysis. Peak tremor was observed as frequencies between 15-25Hz compared to normal movement at approximately 1.4-10Hz. A single exposure to a known tremorgenic dose of lolitrem B (2mg/kg IP) induced measureable tremor for up to 72 hours in some animals. Initially, intoxication with lolitrem B significantly decreased voluntary movement. By 25 hours post exposure a return to normal voluntary movement was observed in this group, despite continuing evidence of tremor. This effect was not observed in animals exposed to the short-acting tremorgenic toxin paxilline. Lolitrem B intoxicated mice demonstrated a random search pattern and delayed latency to escape a 3 hours post intoxication, however by 27 hours post exposure latency to escape matched controls and mice had returned to normal searching behavior indicating normal spatial learning and memory. Together these data indicate that the tremor exhibited by lolitrem B intoxicated mice does not directly impair spatial learning and memory but that exposure does reduce voluntary motor activity in intoxicated animals. Management of acutely affected livestock suffering toxicosis should be considered in the context of their ability to spatially orientate with severe toxicity.
Graphical abstract

Highlights

- Single exposure to lolitrem B toxin (2mg/kg) causes tremor lasting up to 72 hours in mice.
- Quantitation of tremor can be accurately measured in mice exposed to paxilline and lolitrem B using piezoelectric pressure sensor tremor analysis.
- Lolitrem B exerts a negative, but resolving, effect on voluntary movement despite continued presence of tremor.
- Lolitrem B intoxication does not impede spatial learning and memory in mice.
- Acute effects on spatial cognition are likely to contribute to mortality and morbidity in field cases of perennial ryegrass toxicosis.

Keywords
Lolitrem B, paxilline, tremor, spatial learning, memory, paxilline, voluntary movement, coordination.
4.1 INTRODUCTION

Perennial ryegrass toxicosis (PRGT) is a common pasture toxicity that presents in domestic herbivores causing tremor, ataxia and myoclonus. The indole diterpenoid neurotoxin lolitrem B, a large conductance calcium channel (BK) antagonist (Knaus et al. 1994; Nardi and Olesen 2008; Imlach et al. 2009; Imlach et al. 2011; Zhou and Lingle 2014) has been identified to cause the tremor and ataxias (or ‘staggers’) associated in affected animals. Alterations in mentation and / or behaviour have also been observed in PRGT-affected animals (Cunningham et al. 1993). Specifically, hyperaesthesia, allodynia, disorientation and aggression have been noted in sheep, cattle and horses suffering from perennial ryegrass toxicosis (Johnstone 2010; Reed et al. 2011a; Johnstone et al. 2012; Combs et al. 2018). Researchers have also noted a failure to seek shade and mass drowning events in toxin affected animals (Reed et al. 2002; Reed et al. 2011a). Both suggest a herd level effect from cognitive deficits, yet the underlying mechanism of these neurological changes has not yet been defined nor their relationship to tremor investigated.

Evidence from field cases (Gallagher et al. 1982b; Reed 2009; Reed et al. 2011c; Combs M 2014) has suggested that some of the behavioural changes observed in animals suffering from PRGT in the field may be related to deficits in spatial orientation, spatial learning and / or spatial memory. BK channel dysfunction having previously been shown to impair learning in rodents in addition to tremor (Matthews and Disterhoft 2009) and spatial learning specifically (Mirkowski 2013; Typlt et al. 2013) Normal spatial learning and memory are functions governed by the hippocampus, but there is growing evidence that the cerebellum may also play a role (Petrosini et al. 1996; Lalonde and Strazielle 2003; Brandt et al. 2005; Passot et al. 2012). BK channels are strongly expressed in the cerebellum suggesting a causal link between cerebellar and behavioural signs observed in lolitrem B intoxicated animals. Alternatively, differential expression of BK channels across the brain (Augustynnek et al. 2014) as well as differential calcium dependent affinities for BK channel activation in the presence of lolitrem B and paxilline (Imlach et al. 2008) might also be conferring the behavioural effects observed in lolitrem B intoxicated animals.
Both small and large ruminants have been investigated to try to define the neurological changes occurring in animals exposed to naturally occurring neurotoxins under field conditions (Edwards et al. 1996; 1997; Lee et al. 2006; Edwards et al. 2011; Combs et al. 2018c), however, reports on cognitive states have been largely observational and conferring causality to particular cognitive states is problematic. In contrast, rodents are relatively inexpensive to maintain and are accessible for use in well characterised behavioural experimental models known to extrapolate to other species with high level of fidelity (Miwa et al. 2006; Miwa 2007). The tremorgenic toxins harmaline and penitrem A have previously been examined in vivo using rodent models (Montigny and Lamarre 1975; Milner et al. 1995; Breton et al. 1998; Cavanagh et al. 1998; Martin et al. 2005; Miwa et al. 2006).

To test the hypothesis that lolitrem B intoxication may impede normal spatial learning and memory; spatial learning and motor performance was examined in mice exposed to a single intraperitoneal dose of lolitrem B toxin and their tremor, spatial learning and memory and coordination compared to untreated and paxilline-treated controls. The BK channel blocker, paxilline (Smith et al. 1997; Zhou and Lingle 2014) was used as a positive tremorgenic comparator and profiles compared between the two toxins.

4.2 MATERIALS AND METHODS

4.2.1 Animals

The experiments described in this study were approved by Charles Sturt University Animal Care and Ethics Committee (Protocol 11/057) and were compliant with Australian national standards and guidelines for use of animals in experimental procedures. C57Bl6/Ola strain of 4-5 weeks of age (Monash Animal Research Platform, Melbourne, Australia) were used for this study. Animals were group housed in individually ventilated cages (MicroVENT, Able Scientific, Perth, WA) for the duration of the experiment and provided with behavioural enrichment. Standard laboratory rodent pelleted feed and water were available ad-libitum. Animals were maintained under a 12 hour light/dark cycle.
4.2.2 Toxins

Lolitrem B (CAS No. 81771-19-9, provided by S. Finch, AgResearch, Palmerston North, New Zealand) or paxilline (CAS No. 57186-25-1, Sigma-Aldrich, MO, USA) were prepared for intraperitoneal (IP) administration by dissolving in vehicle (9:1 DMSO: dH₂O v/v) using gentle heating (40°C) and agitation. Lolitrem B was formulated to a concentration of 1mg/mL and paxilline 3mg/mL. Individual doses were drawn using a 0.3mL insulin syringe immediately prior to injection. Lolitrem B was administered at a rate of 2mg/kg IP and paxilline at 6 mg/kg IP, based on previously reported dose rates shown to cause overt tremor in mice (Imlach et al. 2008).

4.2.3 Experimental Design

To compare tremor analysis over time, in a preliminary experiment 8 animals were given a dose of either paxilline (6mg/kg paxilline) or lolitrem B (2mg/kg lolitrem) via intraperitoneal (IP) injection to confirm tremor profiles. Times of analysis for comparative spatial, cognitive and tremor were assigned accordingly. Preliminary testing using piezoelectric tremor analysis showed high sensitivity to detect tremor in tremorgenic toxin treated mice. Lolitrem B intoxicated mice showed an increase in tremor motion power ratio (TMPR) from one hour post intoxication to a peak at 6 hours post intoxication after a single IP dose of lolitrem B (2mg/kg, n = 8). TMPR remained constant to 16 hours post exposure after which tremor intensity declined but did not return to pre-intoxication level until 72 hours post exposure. In comparison, paxilline intoxicated animals showed peak tremor at 1.5 hours post intoxication returning to baseline by 4.5 hours (see Appendix 10.2). Similar studies by Imlach, Finch and others, (Finch et al. 2007b; Imlach et al. 2008; Imlach et al. 2009; Imlach et al. 2010; Imlach et al. 2011) confirmed that paxilline exerted a short window of peak effect (approximately 3 hours) whilst lolitrem B exerted a long window of effect (up to 72 hours). Refer to Appendix 10.2, Figure 10-10, 11 and 12.

To examine the impact of lolitrem B exposure on spatial learning and memory and cognitive behaviour in lolitrem B and paxilline intoxicated mice, twenty eight animals were randomly assigned to one of four treatment groups 1) untreated controls (WT); 2) vehicle-only control group that were exposed to 90% DMSO: 10% dH₂O w/v only; (DMSO); 3) lolitrem B treatment group exposed to 2mg/kg lolitrem in vehicle (LOL),
Chapter 4  Characterisation of Neurological Dysfunction induced by the BK Channel Antagonists Paxilline and Lolitrem B using a Mouse Model.

and 4) paxilline treatment group exposed to 6mg/kg paxilline (PAX) via IP injection. N was 7 animals in each case. Spatial learning, cognitive and behavioural changes were compared to tremor intensity in the same animals at various time points over 4 days. Sequence and timing of tremor and behavioural tests are shown in Figure 4-1.

Figure 4-1: Time line of testing over 4 days: Colored arrows denote testing and time. For every group mice performed a sequence of tests daily as shown above. Black arrow at Time 0 indicates time of injection. Blue shaded areas denote dark periods during 12 hour light-dark cycle. NOR, novel object recognition.

4.2.4 Tremor Analysis

A novel tremor analysis was used in this study to accurately quantitate tremor characteristics. Measurement of tremor was undertaken prior to behavioral and cognitive testing to determine tremor severity relative to other measures. To measure frequency and amplitude of tremor associated with intoxication, mice were placed individually into a closed container mounted on a piezo-electric pulse transducer sensor (ADI Technologies, USA). Data from the sensor were converted through an AC amplifier (model DP-311; Grass Instruments, West Warwick, RI) with a bandpass of 1–50 Hz and a sampling rate of 100 samples/second. Motion power output was recorded for 3 minutes and analyzed for change of frequency and power using LabChart™ 7.0 (ADInstruments, Castle Hill, Australia).

Two measures of tremor activity were identified from these data: Peak tremor frequency (PTF) and Tremor-motion power ratio (TMPR). In both cases, Fast-Fourier-Transformation (FFT) converted raw tremor data to a power output spectrum of between 0 and 50 Hz with a sampling size of 1024 [Frequency (Hz) vs. Power (V²)], offering a spectral resolution of 0.01Hz (Figure 4-2).
To assess PTF, FFT spectrums were compared to identify the frequency (Hz) at which the greatest power output occurred ($V^2$). Un-intoxicated control animals showed peak activity defined by a 0 to 10 Hz bandwidth, representing normal movement. Tremor frequencies in of lolitrem B intoxicated mice were between 15 and 25Hz (Figure 4-2 A-H; Appendix 10.2.1, Figure 10-9). By calculating the area under the curve (AUC) of both the peak tremor frequency bandwidth and total tremor profile, tremor intensity was converted to a Tremor-Motion Power Ratio (TMPR) using the calculation [Tremor frequency AUC/ Total AUC (0-50 Hz)].

4.2.5 Coordination and Voluntary Motor Activity

Parallel rod apparatus (ANY-maze™ (Stoelting Co., Wood Dale, IL, USA)Appendix 10.2, Figure 10-15) was used to assess motor coordination as previously described by Kamens and Crabbe (2007). This is a traditional measure of ataxia in rodents. The digital behavioural tracking software (ANY-maze™ Stoelting Co., Wood Dale, IL, USA) monitored movement via a camera placed directly above the box to evaluate distance travelled and speed, with the mouse missing a parallel rod and placing its foot on the steel plate positioned below the rods closed an electrical circuit (a “touch”) thus indicating level of coordination. Number of touches and distance travelled were analysed for each animal at each time point shown in Figure 4-1.
Figure 4-2: Fast Fourier Transformed (FFT) tremor frequencies in lolitrem B (A-D) and untreated control mice (E-H) indicating peak range frequencies for normal movement (<10Hz) and tremor (15-25Hz, marked by dotted lines). Graphs show increase power output in tremor frequencies even at 73 hours (D) where an indicative tremor peak can still be observed with a marginal shift to above 20Hz. In control mice low frequency power output predominates at all time points. See Appendix 10.2, Figure 10-12 for composite FFT from DMSO (vehicle) injected mice.
4.2.6 Spatial Learning and Memory

To assess spatial learning and memory mice were exposed to the Barnes Maze (Barnes et al. 1980; Berta et al. 2007, Appendix 10.2, Figure 10-16) at the same time each day on four consecutive days: at 3, 27 and 51 hours a standard Barnes maze with open escape hole, at 73 hours a probe trial with the escape hole blocked was performed (Figure 4-1). Barnes maze analysis was performed using ANY-Maze™ (Stoelting Co., Wood Dale, IL, USA) apparatus and movement tracking software as previously described. The maze was a 90cm diameter steel disk, with 18, 10cm diameter holes evenly distributed around the circumference. 17 holes led to shallow trays that do not allow the mouse to escape, the 18th hole was the ‘escape hole’. For consistency of stimulus, visual cues were placed around the room and remained constant for each trial and the experimenter sat in the same place for each test. For aversion motivation the room was brightly lit with florescent background lighting and an additional spotlight (100W) over the table. White noise was also played during each trial.

Each test consisted of four trials per animal per day, with each trial spaced 15 minutes apart. At the onset of testing, each animal was placed in the centre of the table (with orientation randomised between trials) and was allowed to freely explore for five minutes. Animals failing to find the escape hole in 5 minutes were gently directed to it and the experiment terminated. The probe trial followed the same testing protocol except that no escape hole was provided. Barnes maze data was analysed for the following measures: Latency to entry of escape hole (seconds); distance travelled (m/s), and time inactive (seconds).

Observations of occupancy plots (the pattern of exploration) was used to aid characterisation of neurological changes with regards to maze searching patterns (Barnes 1979; Barnes et al. 1980). Categorisation of these search patterns was according to a system developed by Bach et al. (1995), with the following modifications: The search pattern designated “random” has been designated “mixed” as mice demonstrate patterns representing a combination of grid and sequential type search patterns. “Random” searching in this study was allocated to animals that do not appear to be following any logical search behaviours by this analysis.
4.2.7 Novel Object Recognition (NOR) Test

The novel object recognition (NOR) test was used to assess visual recognition and contextual memory (Frick and Gresack 2003; Antunes and Biala 2012) as well as affective bias and voluntary movement. Visual recognition and contextual memory rely less specifically on the hippocampus than spatial memory (Frick and Gresack 2003; Antunes and Biala 2012). NOR test was performed using the ANY-Maze™ apparatus and tracking software as described previously. Briefly, animals were placed into a 40cm x 40cm opaque black box containing two of six randomly select novel objects: grey cube, grey sphere, white cube or white sphere and black cube or black sphere. For each test two different objects were placed in the enclosure located towards the centre of the box and oriented to adjacent corners (Appendix 10.2, Figure 10-13). For each repetition of the trial, one object was kept constant and one novel object introduced. Animals were allowed to explore freely for five minutes during which both movement (distance travelled and maximal speed) and interactions with objects were recorded including time spent within a fixed zone around each object or time orientated towards each object.

4.2.8 Statistical Analysis

Statistical analysis of TMPR was performed by two way-ANOVA, with post-hoc analysis performed where indicated with Tukey’s HSD (α= 0.05). For analysis of data captured during NOR and parallel rod tests two way-ANOVA with repeated measures was performed to examine distance, mean speed, maximum speed and the ratio of NOR object to old object interaction and parallel rod touches. For post hoc analysis comparing individual groups, Tukey’s HSD (α= 0.05) was used. Latency to entry of escape (target) hole, time inactive and occupancy plots were analysed for data collected during Barnes maze testing and compared to both treatment group and day. Statistical analysis was undertaken using two way- ANOVA with post hoc analysis with Tukey’s HSD (α= 0.05).

4.3 RESULTS

4.3.1 Piezoelectric Tremor Analysis Identifies Length of Tremorgenic Action of Lolitrem B Toxin in Mice after a Single Exposure

Peak tremor frequency (PTF) and tremor-motion power ratio (TMPR) were analysed after a single toxic exposure at a single timepoint daily for 72 hours to determine longevity of
tremorgenic effect (Figure 4-3) and as an indicator of intoxication and a comparator to analysis of cognition and spatial learning and memory. At 1 hour post exposure, both lolitrem B and paxilline intoxicated animals had increased TMPR compared to vehicle and no treatment controls (*P< 0.001). At 25 hours post exposure only lolitrem B intoxicated animals showed increased TMPR compared to controls (Figure 4-3A, P<0.001).

Peak tremor frequency (PTF) in lolitrem B intoxicated animals was 14.997Hz (SD±2.865Hz) at 1 hour, increasing to 22.573Hz (SD± 1.312) at 25 hours (Figure 4-3B). By 49 hours PFT has reduced to 10.840Hz (SD± 8.666) although this is still increased compared to other groups (P= 0.034) suggesting that tremor is still present in these animals at this time point. By 72 hours post injection, there is no significant difference in PFT between lolitrem B intoxicated mice and the other groups. These data indicate a measureable tremor at 2 days post treatment in lolitrem B animals treated with a single dose of pure toxin.

TMPR and PFT for paxilline exposed animals indicated moderate tremor at 1 hour post exposure (Figure 4-3) with PFT indicative of this effect at 14.0625Hz (SD± 2.497) (Figure 4-3B). Consistent with paxilline conferring a short-duration of action, from 25 hours paxilline intoxicated animals were observed to exhibit PFT frequencies consistent with normal movement (1.2696Hz (SD± 1.151); 49 hours 4.15Hz (SD± 0.313); 73 hours, 1.465Hz (SD± 1.710) (Figure 4-2B) and TMPR was not significantly different to controls (Figure 4-3A).

Together these data indicate that both TMPR and peak tremor frequency are reliable indicators of toxicity with tremorgenic toxin exposure in mice and that lolitrem B has prolonged tremorgenic activity compared to paxilline.
Figure 4-3 Comparison of Tremor Motion Power Ratio (TMPR) and Peak Tremor Frequency (PTF) in lolitrem B, paxilline, vehicle control and no treatment control mice until 72 hours post treatment (n=7 per group). A). TMPR for all groups where hours indicate time post IP injection of toxin. At 1 hour, lolitrem B and paxilline intoxicated animals have increased TMPR compared to DMSO and untreated control groups (*P< 0.001), while at 25 hours only lolitrem B TMPR is increased compared to untreated and vehicle controls (#P<0.001). B) Peak Tremor frequency for animals given a single i.p. injection of lolitrem B or paxilline compared to untreated or vehicle controls. Lolitrem B animals showed increased PTF from 1 to 49 hours but were not significantly different from other groups at 72 hours (*P= 0.02, α P< 0.001, γ P= 0.034). Paxilline has a significantly increased peak tremor frequency at 1 hour only (§ P= 0.04) with no statistical difference at any other time point analysed.
4.3.2 Tremorgenic Toxins Lolitrem B and Paxilline Inhibit Normal Voluntary Movement in Intoxicated Mice after a Single Exposure

To determine if a single treatment affected voluntary motor activity, voluntary movement as was assessed by measurement of distance travelled during the parallel rod (Figure 4-4B) and novel object recognition tests (Figure 4-5B) in lolitrem B and paxilline intoxicated mice. On the parallel rod voluntary movement was strongly inhibited at 1 hour post intoxication, compared to controls in both paxilline (P<0.001) and lolitrem B (P<0.001) intoxicated animals (Figure 4-4B).

At 2 hours post intoxication, voluntary movement was also assessed during the novel object recognition (NOR) test by two measures: distance travelled and maximum speed (Figure 4-4). Similar to results observed for the parallel rod, distance travelled at 2 hours post toxic exposure was significantly reduced for both lolitrem B (P<0.001) and paxilline (P=0.003) treated animals compared to untreated controls (Figure 4-4A). Maximum speed also was significantly reduced at 2 hours post exposure in lolitrem B intoxicated animals compared to controls (P<0.001, Figure 4-4B) but not in paxilline treated animals. At 3 hours voluntary, during Barnes maze testing, movement was also noted to be decreased in lolitrem B intoxicated animals, 1.529 m (SD 0.8), compared to controls, 5.294 m (SD 2.171, P= 0.007). Distance travelled by paxilline intoxicated animals, 4.5 m (SD 1.69) was not significantly less than control animals however time inactive was still increased compared to controls (P<0.001, Figure 4-8).

No significant difference could be identified in either distance travelled or maximum speed between the treatment and control groups on day 2 of testing for either parallel rod or NOR tests (Figures 4-3 and 4-4). For heat-map analysis of movement within the novel object test see Appendix 10.2, Figure10-14. Specifically, voluntary motor activity was not correlated to tremor as measured by PFT and TMPR (Figure 4-3), in lolitrem B intoxicated animals. In lolitrem B intoxicated animals a return to normal movement was observed significantly earlier than a cessation of tremor, in contrast to paxilline treated counterparts where tremor and voluntary movement inhibition appear to be closely aligned.
4.3.3 Lolitrem B and Paxilline Impair Voluntary Movement but Not Motor Coordination

Motor coordination was assessed using the parallel rod apparatus. Number of floor touches, an indicator of loss of motor coordination, was compared between treatment and control groups at 1, 25, 49 and 73 hours post toxin injection (Figure 4-4A). Median number of floor touches was compared between intoxicated animals and controls. Significant differences were observed in the median number of floor touches of paxilline intoxicated animals at 1 hour post injection (P<0.001, Figure 4-4A), consistent with an inhibition of voluntary movement as discussed previously (Figure 3B, 4B). No significant differences in median number of floor touches between groups was identified at any other time point (25, 49 and 73 hours post injection). This suggests that tremor alone is insufficient to cause measurable ill-coordination in lolitrem B intoxicated animals despite measureable tremor at 25 and 49 hours.
Figure 4-4 Measurement of motor coordination in lolitrem B and paxilline intoxicated mice using parallel rod apparatus  A) Loss of motor coordination measured by number of floor touches, and distance travelled, during parallel rod testing. Only paxilline intoxicated animals exhibited significantly fewer parallel rod touches at 1 hour (*P=0.04). No difference was observed between treatment and control groups at 24-73 hours. B) Distance travelled was decreased at hour 1 in lolitrem B intoxicated animals when compared to control (*P<0.001) and in paxilline intoxicated animals when compared to controls (P< 0.001) and vehicle control (§ P= 0.039). No other significant differences were observed in distance travelled at any other time point. Mean± SEM, (n = 7) for all groups.
4.3.4 Paxilline and Lolitrem B Impedes Motor Performance but Not Positive Bias or Exploratory Behaviour in Intoxicated Mice

The NOR test was used to assess visual recognition and contextual memory in lolitrem B and paxilline intoxicated mice. Exploratory behavior (head entry ratio), (maximum speed, m/s) and distance travelled (m) were examined using this apparatus (Figure 4-5). Exploratory behavior (head entry ratio) was not significantly different between treatment groups indicating that intoxication did not impact positive bias under these testing conditions. Only lolitrem B animals showed a reduction in exploratory behavior at 2 hours post intoxication likely due to a significant reduction in voluntary movement (P<0.02, Figure 4-5A). A ratio close to one when comparing head entry ratios between novel and old objects suggests that all animals had no strong preference/ aversion for examining new objects regardless of toxic exposure.

Similar to results described for the parallel rod (Figure 4-4B), distance travelled was significantly reduced in lolitrem B and paxilline treated animals compared to controls at 2 hours post injection, and maximum speed was also significantly reduced in lolitrem B treated animals only (Figure 4-5B, C). Together these data indicated that exploratory behavior is only impeded during initial toxic exposure for both paxilline and lolitrem B animals despite tremor being measureable in lolitrem B treated animals on subsequent days.
Figure 4-5: Analysis of optimistic exploratory behavior (A) and movement (B, C) using the novel object recognition test (NOR). A) Exploratory behavior was significantly reduced in lolitrem B animals only on Day 1 (* P= 0.018). B) Both paxilline and lolitrem B (§ lolitrem B, P<0.0001, * paxilline, P=0.0027) show decreased distance travelled on the first day of the trial when compared to control animals (B). Only lolitrem B shows a difference in maximum speed (* P< 0.0001) when compared to control group animals (C). N = 7 for all cohorts.
4.3.5 Lolitrem B Intoxication Affects Spatial Orientation but Not Spatial Learning and Memory

Spatial learning and memory was analysed using the Barnes Maze (Barnes 1979; Barnes et al. 1980; Berta et al. 2007). At 3 hours post injection of purified toxin, lolitrem B, mice failed to find the escape hole in 27/28 tests and showed a random to a poorly organised mixture of grid and sequential patterns of exploration (Figure 4-6 A-D). Lolitrem B intoxicated mice also showed a significant delay in latency to escape (P<0.001) at 3 hours post intoxication compared to all other groups (Figure 4-7). Latency to escape was not significantly different between treatment groups at any other time point analysed.

![Figure 4-6: (A-D) Barnes maze occupancy plots from four lolitrem B intoxicated mice at 3 hours post intoxication. Pattern of searching is random or shows a weak mix of grid and sequential search patterns. (E-H) Barnes maze occupancy plots from the same mice as shown in A-D at 27 hours post intoxication. Good spatial orientation and sequential/grid searching patterns are now observed. Mouse G appears to have remembered the spatial location of the escape hole, demonstrating good spatial memory despite being unable to find the escape hole unassisted the previous day. Escape hole is identified by double circle, color indicates time spent at location blue (10 seconds)-green-red (>30 seconds).](image)

By 27 hours post injection, lolitrem B intoxicated mice showed significant improvement in search performance employing a range of highly effective search patterns including the ability to employ a “spatial” search pattern implying normal spatial memory function (Figure 4-6 E-H). These data suggest that spatial learning and memory is not impacted by
exposure to the tremorgenic toxins paxilline and lolitrem B but the acute phase of intoxication with lolitrem B impedes searching behaviors.

Figure 4-7: Barnes maze latency to escape hole entry (seconds) for mice 3, 27 and 51 hours after single exposure to lolitrem B or paxilline and compared to vehicle or untreated controls. Only lolitrem B intoxicated animals at 3 hours post injection exhibited significantly increased latency to escape (\( **P<0.001 \)). N = 7 for all cohorts.

Time inactive during Barnes maze testing was also compared between treatment groups. Of the 300 seconds available for each test, lolitrem B intoxicated animals at 3 hours post toxic exposure spent an average of 245.0 (SD 32.61) seconds inactive, significantly more than any other treatment group (\( P<0.001 \), Figure 4-8). By 27 hours post intoxication this trend was reversed, and average inactive time in lolitrem B intoxicated animals was just 71.68 (SD 38.16) seconds, the lowest of all treatment groups, but not significantly different to controls or their paxilline treated counterparts.
Figure 4-8: Barnes maze inactivity (mean ± SE). At 3 hours post injection of toxin, both lolitrem B and paxilline treated animals showed significant differences in inactivity; lolitrem B animals showed significantly increased period of inactivity, whilst for paxilline treated animals the amount of time spent inactive was reduced compared to untreated controls (*P<0.001). At 27 hours post injection lolitrem B mice showed lowest average inactivity time, a significant reduction compared to inactivity of this group at 3 hours (**P=<0.001), but significantly different to any other cohort. No differences were noted between treatments at any other time point analysed. N = 7 for all cohorts. Note: Probe trial (no escape hole) times are shown as the 75 hour data, increased times inactive reflect the lack of an escape route.

4.4 DISCUSSION

This study was designed to investigate behavioural changes induced by a single, acute lolitrem B or paxilline intoxication in rodents. Specifically, this study characterised lolitrem B and paxilline intoxication using measures of tremor, voluntary movement, coordination, learning and memory. The primary hypothesis for the experiments were that lolitrem B intoxication would result in impaired spatial orientation, learning and memory in addition to affecting motor coordination due to anecdotal observations of altered behaviours during naturally occurring outbreaks in other species (Reed et al. 2011a; Reed et al. 2011c; Johnstone et al. 2012; Combs et al. 2014; Combs et al. 2018b).

In order to determine severity of intoxication, a sensitive measure of tremor intensity was developed to allow quantitation of tremor as a measure of intoxication to compare to coordination and behavioural measurements.
4.4.1 Sensitive Quantification and Characterisation of Tremor can be Achieved using Piezoelectric Pressure Sensor Analysis

This study showed that piezoelectric pressure sensor analysis allowed for accurate quantification of tremor patterns both in terms of tremor frequency patterns (FTT analysis and identification of peak tremor frequencies) and analysis of changing patterns of tremor and movement over time (TMPR ratio and observation of FTT spectrums). Fast Fourier transformation (FFT) was used to assess frequency ranges that had consistently increased power output in intoxicated animals correlating to onset and resolution of tremor. Assessment of FFT spectrum both in normal and intoxicated mice enabled identification of frequencies typically associated with movement (<10Hz) and with tremor (15-25Hz) in this rodent model. Tremor motion power ration (TMPR) combine with peak tremor frequency (PTF) proved to be a reliable indicator of degree of intoxication and sensitive enough to detect statistically significant tremor in lolitrem B intoxicated animals up to 49 hours post injection.

The findings from FFT analysis in this study are consistent with other investigations of toxin induced tremor (Gothoni et al. 1983; Lehtinen and Gothoni 1985; Martin et al. 2005; Martin and Handforth 2006; Mansur et al. 2007; Paterson et al. 2009). Analysis of FTT is common as a predictor of tremor although only the current study and Martin et al. (2005) have employed this system to account for variations in both movement and tremor in one measure. In this study the major challenge was that a reduction in movement caused by intoxication with lolitrem B resulted in reduced power output at all frequencies. We therefore identified that that tremor could be quantitated in intoxicated animals that had a significant behavioural motor inhibition response using TMPR and PTF. Data in this study showed that in mice a single IP dose of lolitrem B increased TMPR and PTF up to 49 hours (with some lolitrem B intoxicated mice showing increased readings at 73 hours). This is consistent with other reports of lolitrem B tremor undertaken by observational assessment (Finch et al. 2007b; Imlach et al. 2008; Imlach et al. 2009; Imlach et al. 2010; Imlach et al. 2011). TMPR analysis allowed for a detailed quantitative assessment free of observer bias, an important consideration. In the future these techniques should allow assessment of therapeutics that mitigate lolitrem B or other
neurotoxin-induced tremors, creating a reliable platform for testing of anti-tremor medications for both human and veterinary medicine.

4.4.2 Lolitrem B Intoxication Impairs Spatial Orientation but Not Visual, Contextual or Spatial Learning and Memory

Our study has shown that lolitrem B affected spatial orientation but not spatial learning or memory. At 3 hours post intoxication animals exposed to lolitrem B toxin showed significantly increased latency to escape hole entry compared to controls, with occupancy plots showing lolitrem B intoxicated mice moving in a random pattern and lacking the ability to find the escape hole. This finding supports anecdotal observations in the field that livestock affected by lolitrem B intoxication show significantly poorer spatial orientation, sometimes resulting in death by misadventure. However, at 27 hours post intoxication lolitrem B treated mice showed the lowest average time to locate the escape hole, suggesting that normal spatial orientation had returned. Some mice showed indicators of a spatial searching pattern, indicating memory of the escape hole location (mice previously failing to find the escape hole were gently guided towards it at the end of testing). That this occurred despite significant evidence of continuing tremor in these animals is interesting and suggests that if the acute phase of tremor could be mitigated that these animals could return to normal behaviors quickly. This is an important consideration from a livestock handling and welfare perspective.

Data in this study showed that the ratio of interactions between the novel object and the established object did not differ between any of the treatment groups, except on the first day of testing when lolitrem B mice did not approach the objects due to severe voluntary movement inhibition. These data suggest that lolitrem B did not impact other types of memory (working, short, long, contextual) by our analysis. Together, this study showed that the primary impact of lolitrem B intoxication on rodents in this model is one of voluntary movement inhibition and spatial orientation but not motor learning or memory.

4.4.3 Both lolitrem B and Paxilline Inhibits Voluntary Movement Acutely in Mice after a Single Toxic Exposure Despite Continued Tremor

Our results have showed that lolitrem B intoxicated mice showed significantly reduced voluntary movement during the first testing phase (1-3 hours post injection) compared to
controls suggesting that this was the major inhibiting factor in their performance both during novel object recognition and Barnes Maze testing. After this acute phase, and despite continued evidence of tremor, all spatial performance returns to normal limits. This is the first study to identify that tremor and motor performance are not necessarily correlated in intoxicated animals.

Typlt et al. (2013), in a similar study using a Morris water maze and BK-/− mice (who also tremor), attributed their observation of delayed latency to escape on the first day of testing to slow spatial learning, whereas in this study we have attributed this finding to inhibition of voluntary movement. Our data therefore suggests that inhibition of voluntary movement may also have been exerting a limiting factor on water maze performance in BK-/− knockout mice in previous studies particularly as swimming is a high intensity exercise for rodents with an inherent motor impairment (Typlt et al. 2013). Reductions in voluntary movement have also been noted with the BK channel antagonist penitrem A (Sobotka et al. 1978; Breton et al. 1998), suggestive that these ion channels play a key role in conferring normal movement intention.

A number of explanations for the inhibition of voluntary movement can be postulated. One possible hypothesis is that the inhibition of voluntary locomotion observed in this study is a behavioural response to cerebellar ataxia and vestibular system dysfunction (Finch et al. 2007b; Johnstone 2010; Combs et al. 2018b). Similar to some central tremor disorders, cerebellar tremors are increased with activity (Deuschl et al. 1998) and likewise, ataxia with vestibular dysfunction is worsened by movement and associated with reluctance to move (Balaban and Thayer 2001; Baloh 2003; Brantberg et al. 2005). The interaction between tremor and ataxia needs to be carefully considered in other disease states where both may be present.

In contrast to the mice in this study where voluntary movement is clearly inhibited during acute intoxication, BK-/− mice have been found to have normal motor activity in open field tests and increased exploratory behavior in a Y-maze test (Typlt et al. 2013) suggesting that inhibition of movement in lolitrem B intoxicated animals may occur via a mechanism other than BK channel inhibition. Other research also supports this hypothesis. Sobotka et al. (1978) reported that penitrem A intoxicated mice appeared to have reduced motor activity even at doses that did not illicit tremor and similar
observations were made by Reed et al. (2011a) regarding unusual flock behaviors in sheep sub-clinically affected by perennial ryegrass toxicosis. These findings suggest that BK-related tremor and inhibition of voluntary locomotion may not occur via the same pathways. Other mechanism and/or neurotransmitter pathways may be playing a role, including GABA(A) inhibition, cholinergic activation (Reddy, Quinn et al., manuscript in press) or via intracellular production of neurotoxic free radical species (Berntsen et al. 2013; Berntsen et al. 2017), all of which have been identified to be altered in the presence of tremorgenic mycotoxins.

4.4.4 Lolitrem B and Paxilline Intoxicated Animals Show Inhibition of Locomotion Without Classical Ill-coordination

Motor coordination deficits have been previously reported in paxilline and lolitrem B intoxicated mice and are a key feature of these intoxications (Imlach et al. 2008). Despite being a benchmark apparatus for motor coordination testing in rodents the parallel rod apparatus was more informative in identifying voluntary motor deficits than motor coordination deficits in this study. Previous studies in mice with ethanol-induce ataxia have demonstrated the effectiveness of this assessment tool with increased parallel rod touches in intoxicated animals compared to controls (Kamens et al. 2005; Kamens and Crabbe 2007). The ataxia induced by paxilline and lolitrem B appears to be unique in that, by our analysis, it causes movement inhibition and tremor but not a classic ataxia.

Alcohol, paxilline and lolitrem B are all reported to have a cerebellar component to their movement disorder (Oscar-Berman and Marinković 2007; Imlach et al. 2011), a function that can be clearly identified by parallel rod testing. In contrast, by our analysis, lolitrem B and paxilline intoxicated mice showed significant movement inhibition without ill-coordination despite significant tremor, suggesting the underlying neurological pathways causing this presentation might be more closely linked to those moderating stress, anxiety or other neurological factors (Porsolt et al. 1977;1978; Reed et al. 2011a; Combs et al. 2014). These possibilities warrant further investigation.

4.5 CONCLUSION

The use of a piezoelectric tremor sensor was a sensitive method of detecting tremor in mice. Paxilline and lolitrem B intoxication produced prolonged tremor for up to 72 hours
in lolitrem B intoxicated animals in the 15-25Hz range. Spectral analysis with assessment of tremor frequencies compared to movement related frequencies is a more accurate method for assessing tremor in that it accounts for the role of movement in the production of tremor caused by tremorgenic toxins. Parallel rod apparatus was ineffective in assessing motor coordination deficits in paxilline and lolitrem B intoxicated mice, however it was highly effective at identifying inhibition of exploratory behaviour and locomotion. The mechanism for this inhibition is not clear and warrants further investigation. Profound deficits in spatial orientation, but only during the acute phases of intoxication (less than 25 hours) were identified in lolitrem B intoxicated mice. After this time intoxicated mice matched control in Barnes maze performance, despite ongoing tremor, indicating normal spatial learning and memory. The assessment modalities identified in this study have utility for the assessment of therapeutics for lolitrem B intoxication and potentially other tremorgenic diseases with similar aetiologies.

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Additional material relating to this chapter can be found in Appendix 10.2.
Chapter 5  BROMIDE REDUCES TREMOR AND DECREASES PERCEPTION OF STRESS AFTER A SINGLE EXPOSURE TO LOLITRETM B IN MICE

The aim of this chapter is to test the hypothesis that KBr is an effective therapeutic for reducing the neurological effects of lolitrem B intoxication in mice.

Results from this research provided data for Patent no. US15/038,312; AU2014353885A, Combs M, Quinn J, Edwards S. Prevention and treatment of toxicosis. (Charles Sturt University, James Hagan Court, Boorooma Street, Wagga Wagga New South Wales 2678, Australia) 2015


For full patent see Appendix 10.5: Patent for treatment of toxicosis in sheep.

And

Provisional Patent no. 15/038,261; AU2013904516 Combs M, Quinn J, Edwards S. Stress management in livestock. (Charles Sturt University, James Hagan Court, Boorooma Street, Wagga Wagga, New South Wales, 2678, Australia) 2015


For full patent see Appendix 10.6: Patent for Treatment of Stress in Grazing Animals.

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The results presented in this chapter are currently in preparation for publication in the journal Toxicon.

The findings described in this chapter forms an important link between Chapter 4 which examines neurological deficits induced by lolitrem B and the subsequent large animal studies. It is a detailed examination, in a rodent testing model, of the potential therapeutic potassium bromide for treatment of lolitrem B intoxication. The use of bromide as the
primary therapeutic candidate was based on preliminary work examining a range of potential therapeutics (see Appendix 10.3). These preliminary analyses identified bromide as the primary therapeutic candidate. The experiment described here utilises techniques developed in Chapter 4 for assessment of tremor, coordination and voluntary motor activity to assess efficacy of therapy. In addition c-Fos, a marker of neuronal activation was also used to examine the pattern of forebrain and brainstem activation during exposure with lolitrem B and how this is modified by bromide therapy.

5.1 INTRODUCTION

The findings describe in this chapter explores the use of bromide as therapeutic for lolitrem B intoxication using the validated murine model described in Chapter 4. Bromide as a candidate drug has a number of potential advantages if it were to prove efficacious for treatment of PRGT. Unlike most pharmaceuticals given to sheep, bromide has high oral bioavailability, it also appears to be well tolerated by ruminants and equids and has a cost profile that makes it possible to use over extend periods of time if required (Raidal and Edwards 2008; Quast et al. 2015). Appendix 10.3, Figure 10-17 so comparative voluntary motor activity testing in a number of other candidate drugs.

Bromide has been widely used as a treatment for seizures in humans and animals since the 19th century, but does not appear to have been used for tremor syndromes in humans (Friedlander 1986; Olivieri et al. 1986). Despite this longevity, relatively little is known about its mechanism of action or its effects on neuronal activity. 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 et al. 2008). Anion channels exhibit variable permeability to fluoride, chloride, iodine and bromide (Alexander et al. 2011). The channel’s role is to generate high levels of intracellular chloride and, in the case of neurons, hyperpolarise the cell (Alvarez-Leefmans et al. 1988). In this context bromide appears to have a direct membrane stabilising effect, likely by membrane hyperpolarisation and enhancing GABA –activated currents (Suzuki et al. 1994; Meierkord et al. 2000). Llano et al. (1991) reported that GABA (A) receptors coupled to calcium activated chloride channels play an important role in GABAergic regulation of Purkinje neuron excitability. As bromide has a high affinity for these channels (Robertson 1989), the abundance of GABA receptors within the cerebellum.
makes bromide a plausible therapeutic to counteract a neuronal hyper-excitability in the cerebellum (Bowery et al. 1987).

In the previous chapter a strong inhibition of voluntary movement was identified as a key behavioural outcome of lolitrem B and paxilline intoxication. Changes in forebrain regions affecting control of movement or motivation for exploration are one hypothesis for this reduction in voluntary movement. However, no forebrain lesions have previously been identified in lolitrem B intoxicated animals or animals affected by similar tremorgenic mycotoxins that generate a BK channel blockade (Gilruth 1906; Clegg and Watson 1960; Mackintosh et al. 1982; Breton et al. 1998; Cavanagh et al. 1998; Combs et al. 2014; Combs et al. 2018b). However, lack of histopathological lesion does not necessarily equate with absence of forebrain dysfunction and BK channels are widely distributed throughout the central nervous system and therefore changes in neural function without loss of neuronal integrity could results in the movement inhibition identified in this thesis (Sailer et al. 2006; Sausbier et al. 2006).

c-Fos is the protein product of the immediate early gene c-fos. It is a 380 amino acid protein with a leucine zipper domain for DNA binding and is a well described marker of neuronal activation (Dragunow and Faull 1989; Bullitt 1990; Gao and Ji 2009; Szalóki et al. 2015). Early research revealed that afferent stimulation to the spinal cord and brain increased c-Fos immunoreactivity very rapidly in neuronal cell nuclei, indicative of neuronal activation (Dragunow and Robertson 1987a; Dragunow and Robertson 1987b; Hunt et al. 1987; Dragunow and Faull 1989). c-Fos has also been demonstrated as a reliable marker of neuronal activation in pain and stress pathways (Senba et al. 1993; Harris 1998). Therefore, to examine the mode of action of lolitrem B in inhibition of voluntary movement, mice were examined for c-Fos expression using immunohistochemical staining of frozen sections from perfusion fixed mouse brains of lolitrem B intoxicated animals. c-Fos expression was then compared with neuronal activation patterns in bromide pre-treated, lolitrem B intoxicated mice.

This chapter describes results of experiments designed to test the hypothesis that bromide is effective at reducing the neurological deficits in lolitrem B intoxicated in mice.
5.2 MATERIALS AND METHODS

All animal protocols were approved by the Charles Sturt University Animal Care and Ethics Committee (Protocol No.11/019).

5.2.1 Animals

A total of 37, 4-5 week old, male mice of the C57Bl6/Ola strain (Monash Animal Research Platform, Melbourne, Australia) were used during this study. Animals were housed in groups of four in individually ventilated cages (MicroVENT, Able Scientific, Perth, WA) for the duration of the experiment and provided with behavioural enrichment, standard laboratory rodent pelleted feed and water were available (ad-libitum). Animals were maintained under a 12 hour light/dark cycle under natural spectrum light. Temperature was maintained at 21°C.

5.2.2 Treatments

Mice were assigned to one of the following five treatment groups:

1. Bromide 2700ppm in drinking water for 7 days prior to lolitrem B 2mg/kg IP (n=16); (lolB_Br) (Protocol 1: 11 mice, protocol 2: 5 mice)
2. Lolitrem B 2mg/kg IP (n=12); (lolB) (Protocol 1: 5 mice, protocol 2: 8 mice)
3. Bromide in drinking water at 2700ppm for 7 days (n=9); (Br) (Protocol 1: 9 mice, protocol 2: 0 mice)
4. No treatment prior to testing (n=5). (NT_Con) (Protocol 1: 5 mice, protocol 2: 0 mice)
5. 90% Dimethylsulfoxide (DMSO): 10% dH2O vehicle IP, (n=4). (NT_Veh_Con) (Protocol 1: 0 mice, protocol 2: 4 mice)

Mice were initially assigned randomly to groups of 4 or 5 animals however additional replicates were added to groups 1, 2 and 3 to confirm strongly positive initial findings.

5.2.3 Protocols

Two protocols were used in this study to assess the efficacy of bromide pre-treatment to mitigate lolitrem B intoxication.
In protocol one, animals from treatment groups 1-4 were assessed for tremor, using a piezoelectric pressure sensor, every 60 minutes for six hours after a single injection of lolitrem B injection, 2mg/kg IP. A novel arena and parallel rod testing were undertaken at 210 and 240 minutes post lolitrem B injection respectively.

In protocol two, animals from groups 1, 2 and 5 were tested for tremor using the piezoelectric pressure sensor at 60 minutes post-injection with lolitrem B (2mg/kg) or vehicle (90% DMSO) IP. At 180 minutes post toxin exposure mice were euthanised by barbiturate overdose followed by transcardiac tissue fixation with 4% paraformaldehyde prior to brain dissection for sectioning and c-Fos immunohistochemical staining.

5.2.4 Toxin Formulation

Lolitrem B (CAS No. 81771-19-9, provided by S. Finch, AgResearch, Palmerston North, New Zealand) was prepared for intraperitoneal (IP) administration by dissolving in vehicle (9:1 DMSO: dH$_2$0 v/v) using gentle heating (40°C) and agitation. Lolitrem B was formulated to a concentration of 1mg/mL. Individual doses were drawn using a 0.3mL insulin syringe immediately prior to injection. Lolitrem B was administered at a rate of 2mg/kg IP. Vehicle control comprised 9:1 DMSO: dH$_2$0 v/v.

5.2.5 Tremor Analysis

Tremor analysis in both protocols was performed using a piezoelectric pressure sensor (ADI Instruments, Sydney, Australia.) and PowerLab™ (ADI Instruments, Sydney, Australia) analyser (see Chapter 3 for a detailed description of the apparatus and protocol). Briefly, one minute epochs were selected for fast Fourier transform (FFT) analysis. Severity of tremor was analysed by calculation of power output within the tremor frequency range (10-20Hz) compared to total power output over the measured range (0-50Hz) to formulate a tremor-motion power ratio (TMPR).

5.2.6 Behavioural Testing

In protocol one, at 210 minutes post injection, exploratory behaviours and spatial orientation were assessed by placing mice in an enclosed novel arena with computerised video tracking (Anymaze™, Stoelting Illinois, U.S.A) for three minutes. Animals were assessed for speed and distance moved as well as display of normal exploratory behaviour.
patterns. See Chapter 3 (NOR testing) for a detailed description of the apparatus although in this chapter exploratory behaviour but not object recognition was examined.

5.2.7 Coordination and Voluntary Motor Activity Testing

Coordination and exploratory behaviour was investigated in protocol one using a parallel rod device (AnymazeTM, Stoelting Illinois, U.S.A) and computerised video tracking (AnymazeTM, Stoelting Illinois, U.S.A) at 240 minutes post toxin exposure. Animals were assessed for speed, distance travelled and normal patterns of exploratory behaviour. In addition coordination was assessed using parallel rod apparatus as described previously (see Chapter 3).

5.2.8 c-Fos Immunoreactivity

In protocol 2, three hours after administration of lolitrem B or vehicle, mice were euthanased with sodium pentobarbital (100 mg/kg i.p.), prior to transcardiac perfusion with 20 ml of 0.9% saline, containing 1% sodium nitrite and heparin (5000 I.U./ml), followed by 100 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. Brains were post-fixed for 24 hours in 4% PFA, 0.1MPB, pH 7.4. After repeated washing in phosphate buffered saline (PBS), pH 7.4, brains were placed in 20% sucrose solution overnight prior to preparation for sectioning. Brains were then blocked using a sectioning matrix (Stoelting Co., Wood Dale, IL) and 40 µm coronal sections were cut in four serially adjacent sets using a sliding microtome (SM2000r, Leica, North Ryde, NSW). One series of sections was used for counts meaning that counts were made on sections 160 µm apart. Sections were stored in 0.1% sodium azide in 0.1 M PBS prior to c-Fos immunohistochemistry.

To reveal c-Fos-immunoreactivity (IR). Free floating sections were washed repeatedly in 0.1 M PB (pH 7.4), followed by washing with 50% ethanol containing 3% H₂O₂. Sections were then incubated in 5% normal horse serum (NHS) in PB (pH 7.4) for 30 min. Sections were incubated with rabbit anti-c-Fos antibody (sc-52, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), at 1:5000 dilution with 0.1 M phosphate buffer (pH 7.4; 2% normal horse serum; 0.2% Triton X-100 (PBT-X)), and incubated for 48 h at 4 °C, with gentle agitation. After washing, sections were incubated overnight at room temperature in biotinylated donkey anti-rabbit IgG (1:1000; Jackson Immunoresearch Laboratories,
West Grove, PA, USA) diluted in 2% NHS PBT-X. Sections were incubated for two hours at room temperature in avidin-biotin complex (ABC) reagent (Vector Elite kit: 6 μl/ml avidin and 6 μl/ml biotin; Vector Laboratories, Burlingame, CA, USA).

Immunoreactivity (IR) for c-Fos were revealed by a nickel-intensified diaminobenzidine (DAB) colour reaction. Sections were washed in PB, followed by 0.1 M acetate buffer (pH 6.0), incubated for 15 min in 0.1 M acetate buffer (pH 6.0) containing 2% nickel sulfate, 0.025% 3,3’-diaminobenzidine, 0.004% ammonium chloride, and 0.02% D-glucose. The peroxidase reaction was started by adding 0.2 μl/ml glucose oxidase and stopped using acetate buffer (pH 6.0). Sections were then washed in PB, mounted and coverslipped with DePeX (Merck KGaA, Darmstadt, Germany).

Counts of neurons IR for c-Fos through the rostro-caudal extent of central amygdala (6 sections rostro-caudal each 160 μm apart) and paraventricular hypothalamus (3 sections rostro-caudal, 160 μm apart), using standard light microscopy according to the rat brain atlas (Paxinos & Watson, 1996) by an observer unaware of group allocations. Total number of c-Fos IR neuron were compared between brain regions and treatment groups.

5.2.9 Statistical analysis

Two-way repeated measures ANOVA were used to assess differences in tremor-motion power ratio (TMPR) from piezoelectric pressure sensor recordings. Behavioural data was analysed using a one-way ANOVA, assumptions of equal variance and normality were violated so data sets were log10 transformed. With parallel rod analysis a large number of datapoints were = Ø, therefore Box-Cox transformation was used. The mean total of c-Fos immunoreactive neurons data was analysed using a one-way ANOVA. With all datasets pairwise comparisons were conducted using Tukey HSD post-hoc analysis with α set at 0.05.

Analyses were undertaken using IBM SPSS Version 25.0.0 (IBM, Armonk, USA), and Prism 8.0 (GraphPad Software, La Jolla, USA).
5.3 RESULTS

5.3.1 Oral Bromide Therapy Reduces Lolitrem B Induced Tremor in Rodents

Prior to injection FFT analysis of peak tremor frequency was 2.8Hz (SD= 0.5) indicative of normal movement. Similar to results shown in Chapter 4, for lolitrem B intoxicated mice the FFT analysis of peak tremor frequency showed frequencies of 11.8Hz (SD= 0.5) and 13.0 Hz (SD= 1.6) at 180 and 360 minutes post lolitrem B injection (2mg/kg IP) respectively. Visual comparison of composite FFT waveform between bromide pre-treated mice and control mice at 60 minutes after lolitrem B (2mg/ kg IP) injection revealed a distinct ablation in tremor peak (10-20Hz) in bromide treated mice and an increase in activity in the low frequency portion of the spectrum associated with normal movement (0-10Hz) (Figure 5.1).
Chapter 5  Bromide Reduces Tremor and Decreases Perception of Stress after a Single Exposure to Lolitrem B in Mice

Figure 5-1: Protocol 2: FFT analysis of tremor, at 60min post lolitrem B injection (2mg/kg IP) and no treatment (n=8) or bromide pre-treated (n=5). Bromide pre-treated animals show more low frequency power output (0-10Hz) and the ablation of the distinct tremor peak (10-20Hz) seen in untreated, lolitrem B intoxicated, mice. This clearly indicates that animal within protocol 2 had a typical tremor response and lolitrem B intoxication as the predicted modification of that response by bromide.

TMPR was calculated for all groups using the frequency range of 10-20Hz as indicative of tremor frequencies (figure 5.2), Similar to the results reported in Chapter 4 (Combs et al. 2019) TMPR was elevated for the lolitrem B exposed animals in comparison to un-intoxicated controls at all time points from 1 hour (F (1.384, 8.305) = 22.28, P< 0.01). Compared to un-intoxicated mice, Bromide + lolitrem B treated mice exhibited an elevated TMPR’s in the first three hours post lolitrem B injection, however this only reached the level of statistical significance at one time point, 180 minutes post intoxication (t(6)= 4.349, P=0.01). Lolitrem B intoxicated mice pre-treated with bromide had a lower TMPR when compared to lolitrem B only animals at all time points beyond 1 hour (q(25)= 4.934, P<0.01). (P<0.01). These data indicated that bromide treatment
prior to exposure to lolitrem B resulted in reduced tremor compared to toxin exposed animals not receiving bromide therapy.

![Figure 5-2: Tremor: Motion Power Ratio (TMPR) following lolitrem B injection (2mg/kg IP), (Mean± SEM, lolB-Br n=11, lolB n=4, Br n=9, NT_Con n=5): A main treatment effect is present between groups (*F (1.384, 8.305) = 22.28, P<0.01). Bromide pre-treated animals (n=11) demonstrates a significantly decreased (t(6)= 4.349, P=0.01) TMPR following lolitrem B exposure when compared to untreated, lolitrem B intoxicated, mice. In lolitrem B intoxicated animals that received bromide pre-treatment TMPR is significantly elevated compared to controls only at 3 hours (γ, q(25)= 4.934, P<0.01).](image)

5.3.2 Bromide Therapy Reduces Length of Freezing Episodes and Increases Exploratory Behaviour in Lolitrem B Intoxicated Mice.

To determine whether pre-treatment with bromide could reduce the inhibition of voluntary movement previously identified in lolitrem B intoxicated animals (see Chapter 4, Combs et al. 2019), distance travelled after a single exposure to lolitrem B (2mg/kg IP) in the presence or absence of pre-treatment with bromide was compared. A main treatment effect is present between groups (F (3, 25) = 7.332, P<0.01). Lolitrem B intoxicated mice showed reduced voluntary movement when compared to un-intoxicated
controls (q(25)= 4.451, P=0.02) and lolitrem B intoxicated mice pre-treated with bromide (q(25)= 4.692, P=0.01, Figure 5.3).

**Figure 5-3**: Comparison of distance travelled in novel arena test (Mean± SEM, lolB-Br n=11, lolB n=4, Br n=9, NT_Con n=5): Lolitrem B intoxicated animals pre-treated with bromide (n=11) showed increased voluntary movement when compared to lolitrem B intoxicated animals (n=4) that did not receive bromide (*q(25)= 4.692, P= 0.0138). Lolitrem B intoxicated animals also have reduced movement when compared to untreated control group (q(25)= 4.451, P=0.02). Data log-transformed for analysis. Lolitrem B intoxicated animals pre-treated with bromide were not significantly different to controls.

In mice, freezing behaviour is a defensive behaviour arising from ventrolateral periaqueductal grey circuits and modulated by the central amygdala (Tovote et al. 2016). Between groups there was a strong main treatment effect (F (3, 25) = 8.086, P<0.01). Pretreatment with bromide did not reduce freezing time during the novel arena test in lolitrem B intoxicated animals (Figure 5.4, C). However there was a significant reduction in freezing time per episode (q(25)= 4.131, P= 0.0345, Figure 5.4, B).
Figure 5-4: Freezing behaviour within the novel arena test (Mean± SEM, lolB-Br n=11, lolB n=4, Br n=9, NT_Cn n=5) (A) Lolitrem B intoxicated animals have less freezing episodes than no treatment controls (*q(25)= 6.687, P=0.0004), lolitrem B intoxicated animals treated with bromide likewise had less freezing episode than controls (**q(25)= 4.588, P= 0.164). (B) However, time per freezing episode is reduced in both control groups when compared to lolitrem B intoxicated animals (*q(25)= 5.740, P=0.0023), likewise intoxicated animals pre-treated with bromide have shorter freezing episodes (**q(25)= 4.131, P= 0.0345). (C) This leads to an overall increase in freezing time of lolitrem B intoxicated animals compared to control groups (*q(25)= 4.744, P≤0.0127).
5.3.3 Bromide Treatment Causes Behavioural Disinhibition but does Not Appear to Improve Coordination in Lolitrem B Intoxicated Mice.

Consistent with results shown in Chapter 3, intoxication with lolitrem B inhibited movement and touches on the parallel rod. Main treatment effect was highly significant (F (3, 25) = 8.993, P= 0.0003). Lolitrem B intoxicated animals had no touches recorded in this study, a significant reduction compared to lolitrem B animals pre-treated with bromide (q(25)= 4.041, P=0.0396, Figure 5.5). All animals given bromide (either with or without lolitrem B exposure) had higher numbers of parallel rod touches. Given that the purpose of this test is to measure coordination dysfunction from number of touches, it is difficult to infer improved coordination for this result.

![Graph showing parallel rod touches](image)

**Figure 5-5: Parallel Rod Touches (lolB-Br n=11, lolB n=4, Br n=9, NT_Con n=5):** Lolitrem B intoxication mice (2mg/kg IP) had no parallel rod touches during this experiment, significantly less the than intoxicated animals treated with bromide (*q(25)= 4.041, P=0.0396), bromide control animals (**q(25)=7.157, P=0.0002) and negative control group animals (#q(25)=4.517, P=0.0185).

As movement is a more complex task on the parallel rod apparatus than in open field tests, another measure of coordination on the parallel rod apparatus is maximal rate of voluntary movement. Lolitrem B intoxicated animals showed a reduction in maximal speed when compared to negative control animals (P=0.0256, Figure 5.6). Bromide pre-
treatment did not improve maximal speed to the level of statistical significance when compared to lolitrem B intoxicated animals (Figure 5.6). Bromide treated controls had the highest maximal speed which suggest behavioural disinhibition plays a role in their movement.

**Figure 5-6:** Maximum Speed within parallel rod test (Mean± SEM, lolB-Br n=11, lolB n=4, Br n=9, NT_Con n=5): Lolitrem B intoxicated animals had the lowest maximal speed within the novel test arena. Analysis of log-transformed data revealed an overall treatment effect (F (3, 25) = 7.773, P= 0.0008), pairwise analysis reached the level of significance between lolitrem B and the negative control (*q(25)= 4.693, P= 0.0138) and bromide groups (*q(25)= 5.567, P= 0.0031). Lolitrem B animals pre-treat with bromide had significantly less movement that bromide control, but not not treatment controls (**q(25)= 4.863, P= 0.0104).

**5.3.4 Bromide pre-treatment reduces perception of stress, but not neuro-endocrine pathway responses to lolitrem B intoxication.**

Lolitrem B intoxication generated a pattern of neuronal c-Fos activation and localisation consistent with the activation of stress pathways (Figure 5.7) (Senba et al. 1993). Cell counts were then compared between groups in the central amygdala (CeA) and the paraventricular nuclei (PVN). Bromide pre-treatment in lolitrem B intoxicated animals reduced c-Fos expression in the CeA when compared to lolitrem B intoxicated animals not receiving bromide therapy (q(14)= 5.287, P= 0.005, Figure 5.8, B). The same response was not seen in the PVN (Figure 5.8, A), suggesting that brainstem activation was still
driving a neuro-hormonal response via the PVN, but that perception of physiological stress (via the CeA) was reduced.
Figure 5-7: Photomicrographs showing c-Fos-positive labelling in the paraventricular hypothalamus (PVN), central amygdala (CeA), nucleus tractus solitarius (NTS), ventrolateral medulla (VLM), parabrachial nucleus (PB) and locus coeruleus (LC). 2mg/kg IP lolitrem B (n=8) dramatically increased the number of c-fos-positive nuclei in the PVN, CeA, NTS and VLM compared to vehicle controls (n=4). Scale bar = 200mm.
Figure 5-8: Neuronal cell body counts for c-Fos immunoreactivity: in the paraventricular nuclei (A) and central amygdala (B) (lolB-Br n=5, lolB n=8, NT_Veh_Con n=4). In the paraventricular nuclei, counts of neurons displaying c-Fos immunoreactivity was less in control animals than in animals given lolitrem B 2mg/kg IP (*q(14)= 5.477, P=0.007) and animals pre-treated with bromide and injected with lolitrem B 2mg/kg IP (**q(14)= 4.550, P=0.031) (A). In the central amygdala, vehicle control and bromide + lolitrem B 2mg/kg IP treated animals both have reduced c-FOS expression when compared to lolitrem B 2mg/kg IP treated animals (*q(14)= 6.945, P<0.001, **q(14)= 5.287, P= 0.005), there is no significant difference in c-Fos expression between control and bromide pre-treated animals (B).
5.4 DISCUSSION

5.4.1 Bromide Reduces Lolitrem B Induced Tremor and Improves Mobility of Lolitrem B Intoxicated Mice.

Mice pre-treated with bromide in drinking water prior to injection with a single dose of lolitrem B toxin (2mg/kg IP) showed improved mobility, reduced freezing, and a reduced Tremor Motion Power Ration (TMPR) compared to lolitrem B intoxicated animals without bromide pre-treatment. Specifically, TMPR showed a clear reduction in tremor at all times points beyond one hour post injection. This is the first time that bromide has been demonstrated to be an effective therapy for lolitrem B intoxication in rodents. It is on this rationale that bromide was used for the experiments reported in Chapter 7, using a controlled clinical presentation of perennial ryegrass toxicosis in sheep. Bromide offers considerable promise as a herd level therapeutic both in the acute therapy situation and as a prophylactic agent because of its unique pharmacological properties that include its long half-life, ability to be administered orally or intravenously, physical stability and availability in a price structure that would allow herd level therapy (Quast et al. 2015).

Other postulated drugs include benzodiazepine and barbiturates. Both classes of drugs have significant limitations for use in grazing farm animals, barbiturates in particular have a narrow window of safety and tend to cause profound sedation, recumbency with a significant risk of death (Livanainen and Savolainen 1983; Boothe 1998; Pagel and Parnes 2001; Verster et al. 2004; Tipold et al. 2015). Despite this, examples of use of barbiturates in horses and dairy cattle has been reported, although these drugs were used largely on an individual basis rather than at the herd level or used at low doses to stimulate hepatic enzyme activity rather than for neurological effects (Braud et al. 1971; Bennink et al. 1973; Ravis et al. 1987). In ruminants, benzodiazepines have been used as sedatives and proposed as appetite stimulants, although for herd level therapy of PRGT treatment expense, duration of activity and risk of significant side effects would be concerns (Baile and McLaughlin 1979; Rehm and Schatzmann 1984; Baile and McLaughlin 1987; Abrahamsen 2013). Both drugs are rapidly metabolised in ruminant species increasing cost again and making prophylactic use impractical (Swick et al. 1983; Abrahamsen 2013).
5.4.2 Bromide Reduces Perception of Stress but Not Physiological Stress in Lolitrem

Bromide had a strongly inhibitory effect on neuronal activity in the central amygdala of lolitrem B intoxicated mice. The central amygdala plays an important role in the perception of stress or pain as well as modulating the neuro-hormonal and autonomic response to stress (Davis 1992; Van de Kar and Blair 1999; Neugebauer et al. 2004; Roozendaal et al. 2009; Partridge et al. 2016). Pedersen et al. (2007) demonstrated the injection of a GABAergic drug into the amygdala reduced affective-motivational and sensory-discriminative responses to pain. It seems likely that bromide is having a similar GABAergic effect in the central amygdala and thereby modulating outputs. However, a major neuronal output of the central amygdala is the paraventricular hypothalamus. The lack of response to bromide therapy in the paraventricular nuclei is interesting and suggests the PVN response may be primarily mediated by brainstem activation rather than the central amygdala (Gauriau and Bernard 2002). Stress is known to induce corticotrophin releasing factor (CRF) release in the PVN but the release of both CRF and GABA in the amygdala (Cook 2004; Ji et al. 2007). Partridge et al. (2016) also demonstrated that within the central amygdala CRF+ neurons had GABA as the predominant co-transmitter. It is plausible therefore to suggest that bromide may have greater activity at the central amygdala due to the greater role of GABA in the stress response at the central amygdala.

5.4.3 Bromide Reduces Lolitrem B Inhibition of Movement but Does Not Improve Conscious Proprioception.

Experiments reported in this chapter and in Chapter 4 showed that lolitrem B intoxicated rodents exhibited reduced exploratory movement and had increased freezing behaviours compared to their un-intoxicated counterparts. Defining the exact mechanism by which this occurs is beyond the limits of this study however a hypothesis can be generated taking my findings and previous studies into account. Stress will inhibit natural exploratory behaviours in mice (Bourin et al. 2007) and this is consistent with reports of subclinical lolitrem B intoxication in horses, cattle and deer where animals exhibit excessive behavioural responses to stressful situations (typically animal handling procedures). This
has variably been reported as hyperaesthesia or allodynia (Mackintosh et al. 1982; Miyazaki et al. 2004; Johnstone 2010; Reed et al. 2011c).

Notable in this chapter’s research was that the animals demonstrating the most exploratory behaviour were the bromide control animals. This suggests modulation of the normal fear response to a novel environment (Crawley and Goodwin 1980; Crawley 1985; Prut and Belzung 2003). It should be noted however that Pedersen et al. (2007) did not find an improvement in exploratory behaviour following injection of a GABAergic into the amygdala, although the study was a pain based study (peripheral nerve injury) and so other factors beyond normal behavioural inhibition may have affected movement.

In lolitrem B intoxicated mice pretreated with bromide, the length of freezing episodes was reduced. Freezing behaviour is a defensive behaviour arising from ventrolateral periaqueductal grey circuits and modulated by the central amygdala (Tovote et al. 2016). Reduction in length of freezing episodes suggests improved modulation of freezing behaviour under the influence of bromide. The finding of reduce c-Fos activity in the central amygdala of these animals suggest that bromide is facilitating improved modulation of this behavior within the central amygdala’s neural circuits.

5.4.4 Implications for Bromide Therapy of Tremor Syndromes of Cerebellar Origin

In this study bromide proved effective at reducing stress induced movement inhibition, perception of tremor-associated stress and cerebellar tremor. Movement induced tremor, exacerbated by stress is a feature of the most common form of tremor in humans, namely essential tremor, and several of the drugs used for this treatment of this condition in humans were trialed in lolitrem B intoxicated animals prior to this study (Appendix 10.3). These observations raise two important questions:

A. Is lolitrem B a useful inducible disease model for essential tremor in humans and,
B. Could bromide be a therapy for essential tremor in humans?

Handforth (2016), in a review of animal models for essential tremor, argued that BK channel blockade as induced by lolitrem B showed utility as a secondary model for essential tremor only, useful for research on cerebellar tremor but not essential tremor.
The reasoning for this appears to be primarily around the lack of cerebellar lesions present in the animals exposed to BK channel antagonists. However, the work presented in this thesis demonstrates, this is a misreading of the literature as cerebellar lesions (Purkinje neuronal loss and proximal axonal bodies) have been clearly demonstrated to occur with lolitrem B intoxication (Mackintosh et al. 1982; Combs et al. 2014; Combs et al. 2018b) and penitrem A intoxication (Breton et al. 1998; Cavanagh et al. 1998; Lu et al. 2008; Lu and Parker 2009). Indicating that these intoxication states do accurately reflect the clinical and pathological symptomology of essential tremor in humans. Lolitrem B intoxication should therefore be investigated as another possible disease model for essential tremor.

Bromide was highly effective in this study at ablating tremor and more effective than any other drug used in pre-trial testing (Appendix 10.3). Further examination of the potential of bromide as a therapy for essential tremor should include testing in other animal models of essential tremor, in particular harmaline induced tremor, but also treatment of transgenic mice models with BK channel mutations should be considered to observe the effect of bromide in the absence of these cellular ion channels.

The use of bromide in mammals to treat neuronal excitability was first reported in 1861 and is now common, particularly for treatment of idiopathic epilepsy in dogs (Wilks 1861; Kluger et al. 2009; Baird-Heinz et al. 2012), however bromide therapy in sheep was not reported prior to publication of the work described in this thesis (Chapter 7, Combs et al. 2019). Bromide therapy presents a number of potential benefits to its use in livestock where the complex physiology of the rumen often makes treatment with oral therapeutics a challenge. As bromide is a simple ion it avoids problems with poor bioavailability in ruminants due to digestion and high first pass metabolism (Quast et al. 2015). This allows effective oral administration. Also bromide has a long half-life in ruminants, which should allow for single administration therapy or chronic prophylactic therapy at relatively low cost (Quast et al. 2015).

5.4.5 Conclusion: Bromide is a Potential Therapeutic for PRGT

This chapter clearly demonstrates the efficacy of bromide as a treatment for lolitrem B toxicity, with improved mobility and reduced tremor. The reduction of c-Fos in the central
amygdala with bromide therapy also suggests treatment with bromide confers a significant welfare benefit (reduced perception of stress).

Additional material relating to this chapter can be found in Appendices 3, 5 and 6.
Chapter 6 DEVELOPMENT OF A MODEL FOR INVESTIGATION OF PERENNIAL RYEGRASS TOXICOSIS IN SHEEP

Following the successful identification of bromide as a potential therapeutic for PRGT (Chapter 5), using the murine model of PRGT (developed in chapter 4), the focus of studies moved to testing in sheep. Field testing was not possible due to the unpredictable nature of disease outbreaks in affect areas and the difficult of objectively assessing effect in this setting (Chapter 3). Therefore the aim of this chapter was to develop an induced disease model with in a grazing species for suitable for future therapeutic testing.

This chapter describes the development of a pen-fed laboratory model of PRGT using the most severely affect grazing species, namely sheep. A particularly emphasis of this chapter is attempts to develop a series of objective tests that could be used to grade the severity of disease. Selection of potential testing modalities was based both on observations made, in chapter 3, 4 and 5, and also an examination of the literature (chapter 2). Neurological observations made in Chapter 3, with the distinct change from disorders in limb sequencing (dysdiadochokinesis) to disorders whole limb muscle contraction and relaxation (rhythmic myoclonus) form the basis of gait analysis testing in this chapter. Likewise observations of spatial orientation deficits in Chapter 4 informed the decision to include spatial orientation as part on the gait analysis testing in this chapter.

Observation of stress behaviours in field cases (Chapter 3), as well as activation of neuronal stress pathways (Chapter 5) as well as literature observations of hyperaesthesia or allodynia (Johnstone, 2010) lead to the inclusion of testing for stress (faecal cortisol metabolites) and changes in mechanical nociceptive threshold. Chapters 4 and 5 highlighted the need for an objective measure of tremor. Pressure sensors did not appear feasible in this situation, however previous researchers had suggested that electromyography may be a valid modality for assess tremor, although in previous studies quantitation of waveform was not attempted. Finally perfusion fixation and histopathological analysis of neurological tissue was undertaken, neurological lesions in sheep had not previously been investigated within a controlled laboratory setting and this
was an opportunity to assess the extent of neurological lesions; supporting an important additional aim of this study; to improve understanding of the aetiopathogenesis of PRGT.

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**Development of a model for investigation of perennial ryegrass toxicosis in sheep**

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Chapter 6  Development of a Model for Investigation of Perennial Ryegrass Toxicosis in Sheep

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ABSTRACT

Aims

The aim of this study was to develop a clinical model of perennial ryegrass toxicosis (PRGT) based on feeding a known dose of lolitrem B and ergotamine. With entry to a testing protocol based around a set of clinical criteria rather than set time points thereby producing a consistent clinical presentation for assessment of disease pathophysiology, neurological changes and neuro-histopathology.

Methods

Clinical signs of PRGT were induced in male lambs by ingestion of 0.16 mg/kg LBW/day of lolitrem B toxin and 0.054 mg/kg LBW/day ergotamine. Physiological parameters were monitored throughout the progression of disease including daily clinical examination and monitoring of water intake. Animals entered a two day Testing Phase based on the progression of clinical signs to a level that met set inclusion criteria. Within this protocol neurological signs were monitored using neurological examinations, assessment of gait, surface electromyography (EMG) and mechanosensory nociceptive threshold (MNT). Additionally, serum biochemistry, faecal cortisol metabolites (FCM), full necropsy and histopathological examinations were performed, including assessment of neuropathological changes following perfusion fixation of the brain.

Results

The study model produced typical clinical signs of PRGT including ataxia of vestibulocerebellar origin leading to stumbling as disease progressed. Histopathological characterisation of neurological lesions included the presence of Purkinje cell vacuolation, pyknotic granular layer neurons and proximal axonal Purkinje cell spheroids. Lesions were most apparent within the vestibulocerebellum. Mean Root-Mean-Square (RMS) voltages from triceps EMG at Testing Phase day 2 were significantly increased in treatment group (0.0725mV) compared to control animals (0.0218mV). Decreased
voluntary fluid intake and elevated faecal cortisol metabolites at Testing Phase day 2 correlated with disease progression.

**Conclusions**

Lolitrem B and ergotamine dosing in feed on a live weight basis combined with neurological/gait assessment and perfusion fixation of the brain provides an excellent model for investigation of PRGT and potential therapeutics. FCM may be a useful marker for remote sensing of PRGT-related stress in susceptible flocks.

**Key Words**

perennial ryegrass toxicosis, lolitrem B, tremor, ataxia, sheep, faecal cortisol metabolites

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>PRGT</td>
<td>perennial ryegrass toxicoses</td>
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<tr>
<td>EMG</td>
<td>electromyography;</td>
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<tr>
<td>RMS</td>
<td>Root-Mean-Square</td>
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<tr>
<td>MNT</td>
<td>mechanosensory nociceptive threshold</td>
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<tr>
<td>FCM</td>
<td>faecal cortisol metabolites;</td>
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<tr>
<td>GLDH</td>
<td>glutamate dehydrogenase;</td>
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<td>GGT</td>
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<td>packed cell volume;</td>
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<td>LBW</td>
<td>live body weight;</td>
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<td>CBC</td>
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**6.1 INTRODUCTION**

Plant toxicoses cause major problems for cattle and sheep producers in New Zealand and Australia and can result in significant economic loss in production animal industries annually (Sackett and Francis 2006). PRGT is caused by a variety of toxins produced by the fungal endophyte, *Epichloë festucae var. lolii* (previously *Neotyphodium lolii*) in animals grazing on endophyte-infested perennial ryegrass (*Lolium perenne*) under certain environmental conditions (Gallagher *et al.* 1981; Gallagher *et al.* 1982a; Gallagher *et al.* 1984). The indole diterpenoid, lolitrem B, a potent tremorgenic neurotoxin plays a key role in the clinical presentation of PRGT. When lolitrem B reaches critical levels in
pasture (>1.8ppm), a clinical syndrome, which consists of mild to severe neurological signs, presents in affected flocks and herds (Gallagher et al. 1982b; Combs et al. 2014). Typical clinical signs of PRGT include head tremor and ataxia that is exacerbated by exercise (Cheeke 1995) with spinovestibular cerebellar signs noted including eye deviation (Mayhew 2008). Others have observed that the movement disorder associated with PRGT represents a specific sequence of dyskinesia (Combs et al. 2014). Other clinical signs include ill thrift and diarrhoea, with ergot alkaloids, possibly playing an important synergistic role in production losses through induction of hyperthermia and reductions in feed intake (Cunningham and Hartley 1959; di Menna et al. 1992; Cheeke 1995; Reed et al. 2010; Reed et al. 2011c; Fletcher et al. 2017). Behavioural changes such as erratic or aggressive behaviour, hyperaesthesia or allodynia have also been noted with PRGT by some authors (Johnstone 2010; Combs et al. 2014). 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 et al. 2011; Reed et al. 2011c). A particular feature of PRGT in Australia is the occasional occurrence of high mortality (Reed et al. 2011a). Ergovaline is the principle ergot alkaloid involved in PRGT, however as noted by (Reed et al. 2016) a wide range of ergot alkaloids may collectively contribute to the clinical syndrome. Ergotamine, a vasoactive ergot alkaloid similar in chemical structure and dopaminergic activity to ergovaline but with less potent clinical effect, has also been reported in association with PRGT (Komarova and Tolkachev 2001; McLeay et al. 2002; Reed et al. 2016).

Currently there are no effective on-farm treatments for clinical outbreaks of PRGT. The sporadic nature of the condition makes field testing of therapeutic agents problematic (Reed et al. 2011a; Fletcher et al. 2017). The use of purified lolitrem B injections have been used effectively in mouse models to induce intoxication and allows a multitude of testing modalities to characterise neurological changes, however the clinical presentation with an injectable model, with rapid onset of clinical signs and intense tremor, is at variance to the clinical presentation of naturally occurring cases. In addition, extrapolation of results from therapeutic trials in mice to affected species is limited. Previous large animal feed models, feeding 1.4-3ppm DM lolitrem B, have yielded important neurological and pathophysiological information regarding PRGT (Johnstone 2010, Blythe et al. 2007). However the variable progression of signs of intoxication are
problematic when trying to apply a therapeutic at a set clinical stage of disease (to ascertain efficacy). Additionally, the use of seed from cultivars containing wild type endophyte as the toxin source typically have levels of ergot alkaloids significantly higher than in naturally occurring outbreaks of PRGT. This can lead to clinical signs of ergotism not seen in natural outbreaks that complicate results (Johnstone 2010). This lack of a reliable model has impeded the development of regimens for therapeutic intervention and alleviation of PRGT. The purpose of the current paper was to develop a reliable clinical model of PRGT comparable to the clinical syndrome observed in the field. To achieve this, known doses of lolitrem B toxin and ergotamine were delivered in feed and detailed observations were performed systematically to define the earliest observable neurological signs as well as clinical progression of the movement disorder. In addition entry to an intensive Testing Phase was based around a set of clinical criteria rather than set time points thereby producing a consistent clinical presentation for assessment of disease pathophysiology, neurological changes, coincident neurohistopathology and future therapeutic testing.

6.2 MATERIALS AND METHODS

This study was undertaken with approval from the Charles Sturt University Animal Care and Ethics Committee (Protocol 13/033), Wagga Wagga, New South Wales, Australia. The study commenced in January, 2014

6.2.1 Animals

Animals used were White Sussex x Merino first cross male lambs of between 10-12 months of age (n=18, LBW mean 36.38 (SD 2.79) kg sourced from a single producer. They had been maintained under field husbandry conditions grazing mixed lucerne and wheat stubble pastures before entry to the trial. Before the trial, all animals were clinically normal and had normal plasma biochemical profiling. They were housed in individual pens at the NSW Department of Primary Industries Animal Nutrition Facility, Charles Sturt University, Wagga Wagga and allowed to acclimatise to the environment and handling procedures for 7 days. During this period they were fed a restricted diet of commercially available steam-dried lucerne chaff (Medicago sativa) and had access to water ad libitum. After acclimatisation entry data was collected for clinical examination,
clinical chemistry, gait observations, triceps EMG, and mechanosensory nociceptive threshold testing (MNT).

6.2.2 Treatments

Animals were randomly assigned to either the treatment group (n=9) or the control group (n=9). The treatment group received a set exposure to seed containing lolitrem B and ergotamine. Animals were tested as groups of 6 animals (3 sets of paired animals) with 3 replicates.

Feed containing toxic levels of lolitrem B, a novel endophyte-infested perennial ryegrass seed (Ga66 AR98) was supplied by Grasslanz Technology Ltd, New Zealand. Toxin analysis by liquid chromatography mass spectrometry carried out by AgResearch, New Zealand, showed that the ryegrass seed contained 11.1 mg/kg dry matter lolitrem B and 3.8 mg/kg dry matter ergotamine1. Ergovaline and other alkaloids were not detected.

Ryegrass seed was fed to animals in the treatment group at a proportion of their daily diet sufficient to provide an initial dose of lolitrem B of 0.08 mg/kg LBW/day and ergotamine 0.027 mg/kg LBW/day to acclimatise animals to this unfamiliar diet. After 3 days the toxin dose was increased to 0.16 mg/kg LBW/day and ergotamine 0.054 mg/kg LBW/day. The treatment dose rate was ascertained through estimates of lolitrem B intake on pasture in clinical outbreaks of PRGT (Combs et al. 2014) and extrapolation from other experimental models (Blythe et al. 2007), and was confirmed to induce clinical signs of PRGT by pre-trial testing in a small number of animals. The remainder of the lambs ration consisted of lucerne chaff to give a total ration approximating 2.5% LBW/day (approximately 1kg/day/head). Molasses (Sprengers, Haigslea, Australia) 30% w/v 200 mL was added to improve palatability and aid mixing of ryegrass seed and lucerne chaff to avoid selection. Control group animals were fed a mixture of lucerne chaff (2.5% LBW/day) and molasses, but no ryegrass seed.

All animals were observed twice daily for any adverse clinical signs, with water intake, heart rate and respiration rate monitored at the same time each morning. Observation of neurological clinical signs, including gait assessment, were performed every third day.

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1 Pers. Comm. WJ Mace, AgResearch, Palmerston North, New Zealand
When treatment animals were observed to show moderate gait abnormalities (Table 6-1, rhythmic myoclonus and poor limb sequencing) combined with either stumbling (dropping on hindlimbs or forelimbs) or falling into lateral recumbency, they were transferred from the Feeding Trial into the Testing Phase (see Appendix 10.4, Figure 10-18 for picture demonstrating neurological signs required to meet entry criteria for Testing Phase). The paired lamb from the control group was also transferred at the same time. During the Testing Phase, animals were maintained on the same diet that they were on during the Feeding Trial both for treatment animals and controls.

If, after 20 days on treatment feed, an animal had not shown clinical signs beyond those of mild movement disorder (Table 6-1) the seed ration was increased to provide a lolitrem B dose of 0.22 mg/kg LBW until clinical criteria for entering the testing phase of trial were met. Only two animals in the treatment group qualified for these conditions.

6.2.3 Testing Phase

Following meeting clinical criteria during the Feeding Trial animals entered the Testing Phase protocol which consisted of the following analyses:

Test Day 0: Full clinical examination, gait analysis, faecal sample, urine and blood collection for a complete blood count (CBC), biochemical analysis, faecal glucocorticoid metabolite analysis and urinalysis, neurological examination, EMG and MNT;

Test Day 1: Full, clinical examination, further gait analysis was recorded;

Test Day 2: Full clinical examination, neurological examination including gait analysis, urine and blood collection, EMG, MNT and live weight measurement prior to necropsy.

6.2.4 Clinical signs including observation of gait.

All sheep were subjected to a clinical examination before entering the experiment which included a CBC and biochemical profile, and urinalysis (USG only). A routine in-trial assessment was repeated daily throughout all phases of the experiment and included measurement of respiratory rate by counting thoracic excursion over 30 seconds and heart rate by thoracic auscultation (Littmann Classic II S.E. stethoscope, 3M, St Paul, USA) over 30 seconds. Rectal temperature was measured using a digital thermometer (DT-K01A, Liberty, Scoresby, Australia). General clinical/neurological observations were
also noted on a daily basis; these included observations of nervousness or agitation, changes in normal body movements, limb or head position, observable tremor of the body or head, eye position (strabismus) and movement (physiological/pathological nystagmus), changes in faecal consistency or any other clinical changes worthy of note.

Neurological testing, including proprioceptive testing and cranial nerve examination, was also undertaken on entry (day 0) to the Feeding Trial and Testing Phase days 0 and 2. Gait observations (Table 6-1) were performed by circling animals in yards adjacent to the housing facility and were repeated at three day intervals during the Feeding Trial until gait abnormalities meet the criteria for Testing Phase entry, during which gait analysis was performed daily. Gait analysis was recorded on video on each occasion.

6.2.5 Clinical Chemistry Procedures

Peripheral blood and urine analysis was performed by staff of the Veterinary Diagnostic Laboratory (Charles Sturt University, Wagga Wagga, Australia). Jugular venous blood samples were collected into EDTA and plain tubes for serum biochemical analysis and packed cell volume (PCV). Voided urine for urine specific gravity (USG) was collected by attaching polyethylene bags to the fleece around the prepuce with cyanoacrylate glue (Selleys Supa Glue, Padstow, Australia). Testing was undertaken at three specific time points during the experiment for all animals: 1) Feeding Trial day 0; 2) Testing Phase day 0, 3) Testing Phase day 2 (before necropsy). Tubes were kept chilled until processing and all samples were transported and processed within 60 minutes of collection. USG was determined using a refractometer (VQ5600 refractometer, VetQuip, Sydney, Australia). Biochemical analysis was performed using a Konelab 30i clinical chemistry analyser (Thermo Fisher Scientific, Waltham, USA), using reagents from Thermo Fisher Scientific. PCV measurement was undertaken by placing EDTA blood into capillary tubes (LW Scientific, Lawrenceville, USA) and then centrifuging them at 5000 g for 5 minutes (Heraeus Pico 17 Microcentrifuge, Thermo Fisher Scientific, Waltham, USA); PCV was then read as ratio of whole blood to packed cells. For biochemical analytes a reference range for the experimental cohort was derived from 95% confidence bootstrap interval taken from serum samples of all animals pre-trial.
6.2.6 Measurement of Water Intake and Live Weight Gain

Water intake was measured daily for the duration of the study (both feed trial and testing phase). Animals were weighed (Ruddweigh 700, Gallagher, Hamilton, New Zealand) on day 0 of the Feeding Trial and day 2 of the Testing Phase.

6.2.7 Faecal Cortisol Analysis

Faecal samples were collected from the descending colon of animals during necropsy examination. Faecal samples were lyophilised, pulverised and homogenised, before having a 0.2g aliquot taken for metabolite extraction as previously described (Brown, et al. 1994). Faecal cortisol metabolite (FCM) ELISA was performed as described by Narayan et al. (2013) using anti-cortisol polyclonal antibody (R4866, University of California-Davis, USA). Laboratory validation of FCM ELISA for sheep was undertaken as previously described by Narayan et al. (2013) using an exogenous cortisol standard (#27840; Sigma-Aldrich) (Narayan et al. 2013, Brown et al. 2004). Assay sensitivity is calculated by adding two standard deviations to the mean optical density of zero standards and was found to be 28 pg/ml. Intra- and inter-assay coefficients of variation were determined from internal control samples (30% and 70% bound) included in all assays. Intra-assay coefficients of variation were 1.8% and 5.3% for low- and high- percentage bound controls, and inter-assay coefficients of variation were 5.8% and 1.8%, respectively. All FCM data were expressed as net dry faeces weight basis (ng/g).

6.2.8 Electromyography

Surface electromyography (EMG) traces over the triceps muscle were recorded on Feeding Trial day 0 and on day 0 and day 2 of the Testing Phase. In preparation for recording, the fleece was shaven from an area over the triceps muscle and lanolin removed from the skin using repeated washing in 4% chlorhexidine surgical scrub (Jurox, Rutherford, Australia) and ethanol solution. EMG was undertaken using hydrogel surface electrodes (Kendall, Covidien, Massachusetts, USA), placed 20 mm apart. EMG electrodes had electrode conductive paste (Ten20, Weaver and Company, Colorado, USA) placed on the skin at site of attachment and were attached to the skin using cyanoacrylate glue (Selleys Supa Glue, Padstow, Australia). Standing muscle activity was recorded for a minimum of three minutes using a bioamplifier, (PowerLab,
ADInstruments Dunedin, New Zealand). Data was recorded at a sample rate of 400/s and analysed using LabChart™ software (ADInstruments, Dunedin, New Zealand). A band pass filter was applied at 20-180 Hz and RMS voltages calculated from a 2 minute epoch to assess changes in electrical activity within the triceps muscle (Rohrbach et al. 2014). Animals were standing and unrestrained in pens during recording. Any section of the data recording associated with body movement (e.g. walking) was not used for analysis.

6.2.9 Mechanosensory Nociceptive Threshold Testing

Mechanosensory nociceptive threshold testing (MNT) was undertaken to assess allodynia as a result of lolitrem B intoxication. Testing was undertaken on Feeding Trial day 0, and day 0 and 2 of the Testing Phase. Testing protocol was as previously described (Musk et al. 2014). In brief, a 1.6 mm pressure probe was attached to the hindlimb (left or right as a random selection) with the probe placed over the cranial metatarsus. The fleece was clipped over the region where the probe was to be applied to ensure good probe contact. The attachment cuff and probe (Topcat Metrology, Ely, UK) were applied to the leg during the induction period to ensure familiarisation. During testing, pressure was applied at a rate of 2 N second-1 following a 1N preload (ProdPlus, Topcat Metrology, Ely, UK). End of test was when behavioural response (lifting leg) was elicited; applied pressure at this point was recorded. Five individual measurements were made during each testing period at intervals of 10 minutes. High and low outliers were discarded and the mean of results then used for statistical analysis.

6.2.10 Pathological Examination

Necropsies were undertaken on Testing Phase day 2 and performed at the Veterinary Diagnostic Laboratory, Charles Sturt University, Wagga Wagga. A full clinical examination was performed prior to euthanasia as described previously. At euthanasia, 1000 IU heparin (Wasserberger Arzneimittelwerk, Wasserberg, Germany) was injected into the jugular vein, followed shortly by 163 mg/kg LBW pentobarbitone sodium (Virbac, Sydney, Australia). Immediately after euthanasia, the ventral articulation of C1 was exposed and cerebrospinal fluid collected. The head was then removed and subjected to perfusion fixation with freshly prepared, chilled, 4% paraformaldehyde in 0.1%
phyosphate buffered saline (Sigma-Aldrich, Sydney, Australia). Briefly, the carotid arteries were exposed and a small catheter inserted and ligated to secure the perfusion lines. 0.1% PBS containing heparin (3000 IU/L) was then slowly perfused via the ligated vessels at a pressure of 90 mmHg using a peristaltic pump (Microgon, Laguna Hills, California). Once complete this was replaced with 4% PFA (Sigma-Aldrich, Sydney, Australia) 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°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, processing to paraffin wax (Shandon Excelsior ES Tissue Processor, Thermo Fisher Scientific). All sections were cut at 5µm and stained with haematoxylin and eosin using an automated staining system (Shandon Varistain Gemini ES Slide Stainer, Thermo Fisher Scientific, Waltham, USA) for histopathological interpretation.

Concurrent to fixation perfusion of the head, a routine necropsy was performed. The following tissues were taken for routine histopathology: heart, lung, oesophagus and trachea, liver, spleen, gastrointestinal tract (rumen, abomasum, ileocelecal junction, ileum, caecum and colon), skeletal muscle (gluteal, triceps, tongue and diaphragm), kidney, pancreas, thymus, ileal and cranial lymph nodes as well as tissues from any gross abnormalities observed at time post necropsy. Samples for routine histopathology were fixed in 10% formal saline for a minimum of 48 hours before processing to paraffin wax for routine histopathology.

For histopathology, sections of brain and spinal cord included the following levels/regions: obex, mid cerebellar peduncle, rostral colliculi, thalamus, basal ganglia, hippocampus, internal capsule, pituitary, frontal/temporal and occipital cortex, and cervical, thoracic and lumbar spinal cord. The cerebellum was sampled twice, with one longitudinal full thickness mid sagittal section, and a second sagittal section through the middle of the right cerebellar hemisphere.

6.2.11 Statistical Analysis

For analysis of biochemical data, a simple linear regression was used with Kendall’s correlation analysis applied for non-parametric data sets (bilirubin and USG), parametric
data was compared using Student’s t-test. A mixed repeated measures analysis of variance (ANOVA) was conducted to assess differences in RMS voltages from EMG recordings between the groups and days (Feeding Trial day 0 and Testing Phase day 0 and day 2). Assumptions of equal variance and normality were violated so a logarithmic (Log$^{10}$) transformation was performed on all RMS voltages. Pairwise comparisons were conducted based on estimated marginal means with Bonferroni adjustment for multiple comparisons. Mixed Repeated Measures ANOVA analysis was also conducted to assess different groups and days for heart rate, respiratory rate, temperature and MNT pressures. Pairwise comparisons were conducted with Tukey’s adjustment for multiple comparisons. Water intake and faecal cortisol were analysed using Students t-test. Analysis was undertaken using IBM SPSS Version 20.0.0 (IBM, Armonk, USA), and Prism 7.0 (GraphPad Software, La Jolla, USA).

6.3 RESULTS

6.3.1 Neurological Abnormalities, Gait Disorder and Clinical Changes in Intoxicated Sheep

Treatment group animals demonstrated a clear progression of clinical signs typically associated with on-farm cases of PRGT. None of the animals in the control group showed any clinical signs comparable to those associated with PRGT, however one animal did develop a subtle ataxia. Subsequently histopathological examination revealed an encephalitis (see histopathological result below) and this animal was removed from the study. One animal was excluded from the Treatment group because on review of gait analysis it had not fully met the inclusion criteria for admission to the Testing Phase.

A fine tremor of the head and neck was the first clinical sign of intoxication in treatment group animals, at a median of 7 days (range of onset: 3 days to 10 days exposure to treatment diet), followed by an altered, wide based, stance (median onset 7 days: min=6, max=19 days), truncal ataxia (median onset 17: min=14, max=21 days). Positional ventral strabismus was much more variable in time of onset than other clinical signs (median onset 18 days: min=7, max=33 days).
Gait observations indicated disorders of limb sequencing on rapid movement (dysdiadochokinesia) which occurred relatively early after exposure to the treatment diet, (median onset 8.5 days: min=6, max=20 days) progressing more slowly to rhythmic hyperextension/hyperflexion of limbs (rhythmic myoclonus), (median onset 19.5 days: min=9, max=33 days). Median time to falling in the treatment group was 21 days (min=18, max 34 days). In this group of animals, rhythmic myoclonus always preceded falling and so 18 to 34 days also denotes the minimum and maximum days for entry into the testing phase of the study.

Testing Phase neurological examination showed that menace reflex was either lost or reduced in treatment group animals whilst pupillary light and palpebral reflex remained normal. No other cranial nerve deficits were noted and nystagmus was not noted in any animal in this study. Proprioceptive testing generally appeared normal in the treatment group although position of limb replacement was abnormal in some animals. Treatment animals became increasingly reactive to their surroundings as intoxication increased, particularly to noise.
<table>
<thead>
<tr>
<th>Clinical Stage</th>
<th>Behaviour</th>
<th>Cranial Nerve Changes</th>
<th>Ataxia (Standing)</th>
<th>Tremor*</th>
<th>Gait</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early/ subclinical</td>
<td>Mild nervousness</td>
<td>Occasionally positional ventral strabismus</td>
<td>Subtle wide based stance</td>
<td>Subtle/ intermittent tremor of head/ neck</td>
<td>Normal</td>
</tr>
<tr>
<td>Mild</td>
<td>Nervousness increased reactivity to noise/ movement</td>
<td>Positional ventral strabismus</td>
<td>Truncal sway, wide based stance</td>
<td>Mild tremor: Head, neck, forelimbs, ± body, exacerbated by exercise</td>
<td>Limb sequencing disorder (dysdiadochokinesia) May stumble fore/ hindlimbs, typically into sternal recumbency. Subtle directional deficits, may separate from group.</td>
</tr>
<tr>
<td>Moderate</td>
<td>As for intermediate</td>
<td>Positional ventral strabismus. Loss or decrease of menace response</td>
<td>Wide based stance, limb crossing.</td>
<td>Mild to Moderate tremor: head, neck, forelimbs, body, exacerbated by exercise</td>
<td>Rhythmic hyperextension of limbs, back flexion and neck extension. Falling, often into lateral recumbency. Difficulty directing movement at any pace above walk.</td>
</tr>
</tbody>
</table>

*Tremor observed at rest. Exacerbated by exercise, particularly of forelimbs
Mean temperature, heart and respiratory rates for both treatment and control groups are shown in Figure 6-1. There was no day by treatment interaction effect on temperature (p=0.142). However, a group main effect was present with an increase in the mean temperature in the treatment group compared with the control group (p=0.022). There was a day by treatment interaction effect on heart rates (p<.0005) and respiration rates (p=0.016). Heart rates declined in control animals over the course of the study (p=0.001), but not in treatment animals.
Figure 6-1: Mean (± SEM) (a) rectal temperature (°C), (b) respiration rate (breaths per minute), and (c) heart rate (beats per minute), in lambs fed perennial ryegrass seed containing lolitrem B and ergotamine (grey bars, n=8), and control lambs (white bars, n=8), measured on the first 3 days and last 3 days of the Feeding phase (FP), and the first 3 days of the Testing phase (TP). Differences between groups or times is indicated by *(p<0.01) or ** (p≤0.001).
6.3.2 Clinical Pathology

Results from clinical pathology testing are found in Table 6-2. An increase was observed in Testing Phase day 2 USG compared to Feed Trial Day 0 USG for treatment group animals (p=0.004) and between control and treatment animals for Testing Phase Day 2 USG (p=0.001). No significant elevation of creatine kinase (CK) or aspartate aminotransferase (AST) was observed in treatment group animals, suggesting the absence of any clinically relevant myopathy (Table 6-2).

Analysis of liver function did not reveal clinically significant hepatopathy in any group examined. However, an increase in the liver enzymes gamma glutamyl-transferase (GGT, p=0.004) and glutamate dehydrogenase (GLDH, p=0.045) was observed in treatment animals over the trial. For a full record of clinical pathology results see Appendix 10.4.3-10.4.5
### Table 6-2: Mean (± SEM) serum biochemistry analyte, packed cell volume (PCV) and urine specific gravity (USG) values in control and treatment group animals; Feeding Trial day 0 to Testing Phase day 2 (upper and lower rows respectively), n=8 for both groups. P-values represent significant changes within groups.

<table>
<thead>
<tr>
<th>ANALYTE</th>
<th>CONTROL GROUP</th>
<th>TREATMENT GROUP (LOLITREM B)</th>
<th>REFERENCE RANGE&lt;sup&gt;#&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>USG</td>
<td>1.0248 (± 0.04)</td>
<td>1.0223 (± 0.03)</td>
<td>1.0218 – 1.0287</td>
</tr>
<tr>
<td></td>
<td>1.0142 (± 0.03)</td>
<td>1.0381 (± 0.04)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>tb, ua</td>
<td></td>
</tr>
<tr>
<td>PCV</td>
<td>35.10 (± 0.97)</td>
<td>34.42 (± 1.04)</td>
<td>0.29 – 0.40 l/l</td>
</tr>
<tr>
<td></td>
<td>35.57 (± 0.84)</td>
<td>37.71 (± 2.19)</td>
<td></td>
</tr>
<tr>
<td>UREA</td>
<td>8.41 (± 0.51)</td>
<td>8.68 (± 0.41)</td>
<td>5.4 – 11.4 mmol/L</td>
</tr>
<tr>
<td></td>
<td>7.63 (± 0.26)</td>
<td>6.04 (± 0.34)</td>
<td></td>
</tr>
<tr>
<td>CREATININE</td>
<td>69.8 (± 2.42)</td>
<td>68.5 (± 1.82)</td>
<td>44 – 72 mmol/L</td>
</tr>
<tr>
<td></td>
<td>74.7 (± 3.45)</td>
<td>84.6 (± 4.26)</td>
<td></td>
</tr>
<tr>
<td>TOTAL SERUM PROTEIN</td>
<td>65.11 (± 2.66)</td>
<td>67.88 (± 7.27)</td>
<td>59 – 80 g/L</td>
</tr>
<tr>
<td></td>
<td>68.00 (± 2.44)</td>
<td>69.22 (± 5.38)</td>
<td></td>
</tr>
<tr>
<td>ALBUMIN</td>
<td>32.0 (± 0.50)</td>
<td>32.0 (± 0.45)</td>
<td>29 – 41 g/L</td>
</tr>
<tr>
<td></td>
<td>36.0 (± 0.69)</td>
<td>35.0 (± 0.76)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>wb</td>
<td>xb</td>
<td></td>
</tr>
<tr>
<td>GGT</td>
<td>46.0 (± 2.34)</td>
<td>56.11 (± 1.82)</td>
<td>47 – 95 U/L</td>
</tr>
<tr>
<td></td>
<td>49.3 (± 2.07)</td>
<td>65.22 (± 2.52)</td>
<td></td>
</tr>
<tr>
<td>GLDH</td>
<td>6.22 (± 1.46)</td>
<td>6.33 (± 1.00)</td>
<td>0 – 23 U/L</td>
</tr>
<tr>
<td></td>
<td>16.11 (± 6.11)</td>
<td>36.22 (± 12.76)</td>
<td></td>
</tr>
<tr>
<td>BILIRUBIN</td>
<td>2.22 (± 0.44)</td>
<td>2.11 (± 0.33)</td>
<td>2 – 3 µmol/L</td>
</tr>
<tr>
<td></td>
<td>2.22 (± 0.44)</td>
<td>1.78 (± 0.66)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>: comparison of control and treatment groups Testing Phase day 2.  
<sup>b</sup>: comparison of group Feeding Trial day 0 to Testing Phase day 2.  
<sup>t</sup>,<sup>u</sup>,<sup>v</sup>,<sup>w</sup>,<sup>x</sup>,<sup>y</sup>,<sup>z</sup>,<sup>#</sup>, Reference range for the experimental cohort was derived from 95% confidence bootstrap interval taken from serum samples of all animals pre-trial.  
Gamma-glutamyl transferase (GGT), Glutamate dehydrogenase (GLDH).  

136
6.3.3 Water Intake and Weight

There was no across trial difference in mean daily water intake during the Feeding Trial between the control group, 4.01 (SD 1.7) L and the treatment group, 3.8 (SD 2.2) L. However as intoxication progressed, there was a significant decrease in comparative water intake between intoxicated animals and their control counterparts. Mean voluntary water intake during the testing phase of the study for the treatment group 2.3 (SD 0.36) L was less than in control group animals 3.1 (SD 0.54; p=0.002) L indicating that increasing intoxication resulted in decreased voluntary water intake. There was no significant difference in body weight observed between the two groups over the duration of the trial (p=0.123) with mean weight gain for the control group being 0.079 (SD 0.026) kg/day and 0.032 (SD 0.077) kg/day for the treatment group.

6.3.4 Faecal Cortisol Analysis

FCM levels were higher in treatment group animals compared to controls on Testing Phase day 2 (p=0.02, Figure 6-2). One animal in the control group did not have faeces within the descending colon and so no faecal cortisol analysis could be performed.
Figure 6-2: Mean (± SEM) concentrations of cortisol metabolites (FCM) in faeces collected at necropsy from lambs fed perennial ryegrass seed containing lolitrem B and ergotamine (Treatment, n=8), and control lambs (n=7). Mean concentrations differed between groups (p<0.02).

6.3.5 Surface Electromyography

RMS voltage calculation of triceps muscle activity during standing indicate significant main effects of day, (p<0.001) and of group (p=0.002). However the main effects are qualified by an interaction between days and groups, (p=0.008). Pairwise comparisons conducted based on the estimated marginal means with Bonferroni adjustment for multiple comparisons showed that there was no significant difference between the three test days within the control group. In the treatment group RMS voltages increased between Feeding Trial day 0 and Testing Phase, day 0 (p=0.004), and Feeding Trial day 0 and Testing Phase, day 2 (p<0.001), there was no significant difference between Testing Phase days 0 and 2 (p=1.000) (Figure 6-3).
Figure 6-3: Geometric mean (with 95% CI) root mean square voltage of surface electromyograms recorded from the triceps muscle of lambs fed perennial ryegrass seed containing lolitrem B and ergotamine (grey bars, n=8), and control lambs (white bars, n=8), measured on Day 0 of the Feeding phase (FP), and Day 0 and Day 2 of the Testing phase (TP). Differences between groups or times is indicated by * (p<0.01) or ** (p≤0.001).

6.3.6 Mechanosensory Nociceptive Threshold Testing

Within the treatment group MNT was increased Test Phase day 2 compared to Feeding Trial day 0 (p=0.018) (Figure 6-4). Mean MNT pressures also increased in treatment group animals compared to their control counterparts, however this increase did not reach the level of significance (p=0.088) (Figure 6-4).
Figure 6-4: Mean (± SEM) mechanosensory nociceptive threshold (MNT) recorded in lambs fed perennial ryegrass seed containing lolitrem B and ergotamine (grey bars, n=8), and control lambs (white bars, n=8), on Day 0 of the Feeding phase (FP), and Day 0 and Day 2 of the Testing phase (TP). *Values differed between Days (p=0.019).

6.3.7 Histopathological Changes

Histopathological changes in treatment group animals at necropsy resembled those observed in field cases of PRGT (Parton K 2006; Combs et al. 2014) with some additional features (Figure 6-5). In the treatment group, histopathological lesions in the central nervous system were restricted to the cerebellum and were characterised by the presence of granular layer spheroids, (Purkinje neuron proximal axonal swellings), pyknotic granule cell neurons and intraneuronal vacuolation of Purkinje neuronal cell bodies. Spheroids and granule cell pyknosis occurred in 7/8 and 8/8 treatment group animals respectively whereas these lesions were absent from control group animals. Purkinje neuron vacuolation occurred in 5/8 treatment group animals, one control group animal also had rare Purkinje neuron vacuolation. Lesions were located throughout the
cerebellum, however there was an increased lesion frequency in the region of the flocculus and paraflocculus (Figure 6-5).

One animal in the control group was excluded from further analysis on the basis of histopathological changes. Scattered areas of gliosis in the rostral colliculi and basal ganglia, accompanied by lymphocytic perivascular infiltrate were consistent with an underlying encephalitis.

Other clinically significant gross pathological or histopathological lesions were absent from all animals involved in this study.

![Image: Photomicrograph of a section of the vestibulocerebellum of a lamb fed perennial ryegrass seed containing lolitrem B and ergotamine, showing spheroids (*) and pyknotic nuclei (red arrowheads) present in the granular layer and vacuolation within Purkinje neurons (white arrow head). (H&E, bar=50 μm).](image)

**Figure 6-5:** Photomicrograph of a section of the vestibulocerebellum of a lamb fed perennial ryegrass seed containing lolitrem B and ergotamine, showing spheroids (*) and pyknotic nuclei (red arrowheads) present in the granular layer and vacuolation within Purkinje neurons (white arrow head). (H&E, bar=50 μm).

### 6.4 DISCUSSION

This study clearly demonstrates the utility of PRGT assessment based on clinical stage rather than set time points. Subsequent to this a number of testing modalities have proven particularly useful in the objective assessment of PRGT including standing surface EMG
of the triceps muscle, histopathological changes in the cerebellum and standardised neurological assessment, particularly of gait changes.

The neurological signs reported in this study represented disease progression from early to moderate PRGT in terms of its neurological presentation. Although there was a large variation between animals with regard to timing of onset of neurological changes; within each animal changes followed a consistent progression of signs. Selection of animals for testing based on clinical stage therefore allowed for observation of animals with a consistent set of neurological signs which enhanced the study’s ability to make valid neurological comparisons. Onset of clinical signs in this study was similar to that reported in a previous equine model of the disease where the earliest neurological signs to be observed typical of a vestibulocerebellar lesion (Johnstone et al. 2012). Progression from mild to moderate clinical signs was prolonged compared to that reported by Johnstone et al. 2012 and neurological signs of spinocerebellar dysfunction, such as dysdiadochokinesia, dysmetria and myoclonus, became prominent more slowly. However a cattle model of disease reported moderate neurological changes in animals receiving 1.95 mg/kg dry weight lolitrem B in ryegrass straw at around 3 weeks which is similar to the findings in our study (Blythe et al. 2007). These findings suggest species-specific differences in their sensitivity to orally ingested lolitrem B.

This study reports the first controlled observations of neuro-histopathological changes associated with the pathogenesis of PRGT 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 treatment group animals whereas control animals had no changes (expect one animal with rare vacuolation of Purkinje cells). These findings strongly suggest that this set of cerebellar histopathological changes was primarily caused by exposure to toxic feed in this study and should aid to clarify what has previously been a controversial diagnostic area, by confirming presence of spheroids and cerebellar granular cell pyknosis (leading to reduced granular layer cellularity) as two key diagnostic features of PRGT in sheep.

Histopathological lesions were most prevalent in the vestibulocerebellum, the flocculus and paraflocculus (Figure 6-5). This is one of the most primitive regions of the brain and is involved in autonomic reflex activity, particularly related to balance. These regions
receive major innervation from the vestibular nuclei and play an important role in controlling eye movements, with lesions in this area of the brain typically producing signs similar to vestibular disease (Kheradmand and Zee 2011). Vestibular ataxia has been previously postulated as a feature of the early stages of lolitrem B intoxication in horses (Johnstone 2010; Johnstone et al. 2012). The prevalence of lesions in the vestibulocerebellum may be useful in differentiating PRGT from other causes of cerebellar spheroids in livestock.

Within the brain, the early predominance of vestibulocerebellar signs and correlating distribution of cerebellar pathology observed in the brains of treatment group animals suggests that this region may have a higher sensitivity to lolitrem B toxin. Alternatively, the highly lipophilic nature of lolitrem B would suggest a low bioavailability and slow mobilisation from seed in the rumen. It is therefore possible that the early vestibulocerebellar signs observed in our intoxicated animals are due to more bioavailable intermediates of the lolitrem B metabolic pathway which are more readily solubilised (Munday-Finch 1997). Further metabolomics analysis would be required to confirm this hypothesis.

Animals in this study did not become pyrexic, despite exposure to ergot alkaloid in the treatment diet. However, statistically significant increases in temperature and respiration did occur in treatment animals. The fact that these increases were associated with a clinical peak of lolitrem B intoxication raises a number of interesting questions about the relationship between ergot alkaloids and lolitrem B in cases of PRGT. It is known that both ergot alkaloids and lolitrem B are involved in increasing peripheral vascular tone and therefore a synergistic effect on core temperature regulation in PRGT is likely (McLeay and Smith 1999; McLeay et al. 2002). Results in this study suggest neurological synergism of effect would continue to warrant further investigation.

Objective neuromuscular assessments, MNT and surface EMG both increased day 0 Feeding Trial and day 2 of Testing Phase in treatment group animals (Figures 6-3 and 6-4). For EMG this result was not unexpected as it reflects increasing muscle activity with increasing tremor and ataxia, particularly in muscles such as the triceps, given their role in maintaining normal body position. EMG at this location therefore appears to be a useful measure of neurological changes associated with PRGT. However although Feeding Trail
day 0 MNT results are not dissimilar to those previously reported in sheep (Musk et al. 2014), the day 2 Testing Phase result was contradictory to that hypothesised as animals affected by PRGT have previously been reported to be hyperaesthetic (Mackintosh et al. 1982; Johnstone et al. 2012), suggesting that MNT testing would demonstrate increasing sensitivity to noxious stimuli with increasing intoxication. This was not found to be the case. It is possible, however, that the increased MNT observed in lolitrem B intoxicated animals in this study more reflects their reluctance to move due to ataxia rather than any change in sensitivity to the stimuli. We observed an increased sensitivity to auditory stimuli with increasing intoxication, possibly reflecting large conductance calcium activated potassium channel (BK channel) blockade resulting in auditory temporal imprecision (Johnstone 2010).

It has previously been suggested by us, and others, that dehydration may play a role in the pathogenesis of PRGT in the field, particularly of relevance to severe outbreaks of the disease in Australia where high ambient temperatures may contribute to high morbidity and mortality (Reed et al. 2011a; Combs et al. 2014). The increased USG observed in treatment group animals indicates the kidneys were attempting to preserve water, presumably because of reduced total body water. Decreased fluid intake was also observed in treatment group animals as intoxication reached clinically significant levels, a likely cause of the increased USG. However it cannot be ruled out that fluid loss via gastrointestinal mechanisms was also occurring in these animals (Smith et al. 1997; McLeay et al. 1999). Despite increased USG and reduced fluid intake, clinical dehydration was not present in the animals in this study and elevations in creatinine, albumin and PCV although present, did not reach the levels of clinical significance.

The finding that faecal cortisol metabolites were elevated with intoxication when compared to control animals demonstrates the potential usefulness of this non-invasive modality as a marker of PRGT in flocks with a known history of PRGT. Cortisol is a systemic marker of stress in animals; further assessments in the field are required to assess whether faecal cortisol analyses may prove useful as an early warning tool for farmers who could then institute altered management practices or therapeutic interventions prior to the manifestation of severe clinical signs of PRGT. Chronic elevation of cortisol is also
known to effect productivity in sheep (Knott et al. 2010) and the role of elevated cortisol in PRGT related production losses warrants further investigation.

In conclusion, the use of a set dosing regimen designed to mimic intake of toxin on pasture, combined with entry in to a Testing Phase based on clinical criteria rather than set time points appears to be an effective laboratory model for investigating what otherwise can present as a highly variable disease. Assessment of gait changes using set criteria and RMS voltages from superficial EMG of the triceps muscle appear to be useful tools for the assessment of the severity of neurological changes with PRGT. Further research into the individual toxin activities and potential synergistic activity is warranted.

6.5 ACKNOWLEDGEMENTS

The authors wish to thank Cate Chandler, Alice Birckhead, Emily Birckhead the staff of the Veterinary Teaching Hospital and the Veterinary Diagnostic Laboratory, Charles Sturt University, for their excellent technical assistance during this study. Thanks to G Musk for assistance with nociceptive threshold testing and equipment. JCQ was supported by funding from Meat and Livestock Australia (B.AHE.0233) and a Research Fellowship from the Graham Centre for Agricultural Innovation, Charles Sturt University. MDC was supported by a technical assistance grant from Meat and Livestock Australia.

Additional material relating to this chapter can be found in Appendix 10.4.
Chapter 7  TREATMENT WITH POTASSIUM BROMIDE MITIGATES ATAXIA AND REDUCES TREMOR IN LAMBS WITH PERENNIAL RYEGRASS TOXICOSIS

The preceding chapter in this thesis dealt with the investigations into the development of a model that could be used to improve the understanding of the aetiopathogenesis of PRGT in sheep and to provide a platform for future testing of therapeutic agents. In this chapter the therapeutic agent potassium bromide (KBr) is tested as a treatment for PRGT. There are two main treatment groups in this study, a group that receives daily KBr prophylactically and a group that receives a single dose of KBr after meeting criteria for a specific clinical stage of intoxication. Assessment of surface electromyograms over the triceps muscle forms an essential objective measure of response to therapy, alongside a composite scale of gait assessment, faecal cortisol levels and histopathological examination.

An important adjunctive to this research is the pharmacokinetic studies undertaken within our research group (Appendix 10.6), the studies formed the basis of selected dosages in this chapter (Quast et al. 2015).

Data from this chapter was used in the development of Patent no. US15/038,312; AU2014353885A, Combs M, Quinn J, Edwards S. Prevention and treatment of toxicosis. (Charles Sturt University, James Hagan Court, Boorooma Street, Wagga Wagga New South Wales 2678, Australia) 2015


To view full patent see Appendix 10.5: Patent for treatment of toxicosis in sheep.

And

Provisional Patent no. 15/038,261; AU2013904516 Combs M, Quinn J, Edwards S. Stress management in livestock. (Charles Sturt University, James Hagan Court, Boorooma Street, Wagga Wagga, New South Wales, 2678, Australia) 2015

Treatment with Potassium Bromide Mitigates Ataxia and Reduces Tremor in Lambs with Perennial Ryegrass Toxicosis


This paper has been published in the *New Zealand Veterinary Journal*.

Thus this chapter appears here in its published format.

Scientific Article

**Treatment with potassium bromide mitigates ataxia and reduces tremor in lambs with perennial ryegrass toxicosis**

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To view full patent see Appendix 10.6: Patent for Treatment of Stress in Grazing Animals.
ABSTRACT

Aims

To assess the use of potassium bromide (KBr) as a therapeutic intervention for perennial ryegrass toxicosis (PRGT) in lambs fed ryegrass seed containing lolitrem B.

Methods

Male lambs aged 10–12 months (n=43) were assigned to received ryegrass seed containing lolitrem B, at a dose of 0.16 mg/kg/day (Groups 2, 3 and 4), or lucerne chaff and molasses (Groups 1 and 5). Lambs in Groups 2 and 3 were observed for clinical signs and gait changes until pre-defined signs of PRGT were observed, at which stage they were transferred, with lambs in Group 1, to the Testing phase of the trial. Lambs in Group 3 were then treated with a single oral dose of 300 mg/kg bromide. Lambs in Groups 4 and 5 received KBr daily from the start of the trial (540 mg/kg bromide over 3 days then 20 mg/kg bromide daily) and were transferred to the Testing phase after 18 days. Clinical examination, gait assessment, and surface electromyography of the triceps muscle, with measurement of root-mean-square (RMS) voltages, were carried out on Days 0, 1 and 2 of the Testing phase followed by necropsy, histopathological examination, measurement of concentrations of bromide in serum and CSF, and faecal cortisol metabolites (FCM).

Results

In Group 3 lambs, mean composite gait scores decreased between Testing phase Day 0 and Days 1 and 2 (p<0.001), but increased in lambs in Group 2 between Day 0 and Day 2 (p=0.015). Scores for lambs in Group 3 on Day 2 were lower than for lambs in Group 2 (p<0.001). Mean RMS voltages on Day 2 were higher in lambs in Group 2 than Group 3 (p=0.045). Mean concentrations of bromide in serum were > 800 µg/mL in lambs in Groups 3 and 4 on Day 2. Concentrations of FCM were higher in lambs from Group 2 than in Groups 1 or 5, but were similar in Groups 2, 3 and 4. Histopathological findings in the cerebellum of lambs from Groups 2, 3 and 4 were similar, showing pyknosis of neurons within the granular layer of the cerebellum and proximal axonal spheroid formation in Purkinje neurons.
Conclusions and Clinical Relevance

A single oral dose of 300 mg/kg bromide in lambs with neurological signs of PRGT resulted in reduced composite gait scores and reduced RMS voltages, indicating a significant improvement in clinical signs of ataxia, movement disorder and muscle tremor associated with the neurotoxic effects of lolitrem B.

Key Words

perennial ryegrass toxicosis, lolitrem B, potassium bromide, tremor, ataxia, sheep

BK Calcium activated potassium
CSF Cerebrospinal fluid
EMG Electromyography
FCM Faecal cortisol metabolites
GABA Gamma-aminobutyric acid
LBW Live bodyweight
PRGT Perennial ryegrass toxicosis
RMS Root-mean-square

7.1 INTRODUCTION

Perennial ryegrass toxicosis (PRGT) causes significant economic losses for cattle and sheep producers throughout New Zealand and Australia. The disease is caused by a mixture of toxins including lolitrem B, an indole diterpenoid produced by the fungal endophyte *Epichloë festucae var. lolii* (*Neotyphodium lolii*) (Philippe 2016). Lolitrem B is a potent tremorgenic neurotoxin that is thought to exert its neurological effects via blockade of Ca activated potassium (BK) channels (Imlach *et al.* 2011). BK channels are widely distributed in the central nervous system but are found in high concentrations within the cerebellar molecular layer and within Purkinje neurons as well as in the vestibular system and deep cerebellar nuclei (Sausbier *et al.* 2006; Kaufmann *et al.* 2009). Lolitrem B-induced BK blockade is believed to decrease cerebellar inhibitory output...
resulting in the cerebellar ataxia typically observed in clinical cases of PRGT (Sausbier et al. 2004, Johnstone et al. 2012).

Potassium bromide (KBr) has been used for its neuroactive properties since the 19th century, primarily to treat seizures (Friedlander 1986). The use of KBr in mammals to treat neuronal excitability was first reported in 1876 and is now common, particularly for treatment of idiopathic epilepsy in dogs (Kluger and Malik 2009, Baird-Heinz et al. 2012), however KBr therapy in sheep is previously unreported. As a simple ion, bromide is taken across the cell membrane through the same Ca-dependent ion channels that generate chloride gradients (Podell and Fenner 1993), the role of which is to generate a high concentration of intracellular chloride thus hyperpolarising the cell (Alvarez-Leefmans et al. 1988). \textit{In vitro} bromide has a greater hyperpolarising effect on neurons than chloride and enhances gamma-aminobutyric acid (GABA)-activated currents resulting in generalised neuronal inhibition (Suzuki et al. 1994; Meierkord et al. 2000).

High concentrations of GABA receptors are found within the cerebellum with GABA(B) receptors expressed at higher concentrations in the molecular layer (on Purkinje dendrites) than any other region of the central nervous system and GABA(A) receptors expressed at higher concentrations in the granular layer than any other region bar the frontal cortex (Bowery et al. 1987; Somogyi et al. 1989). The presence of such high concentrations of GABA receptors in the cerebellum suggest that KBr may reduce afferent inputs to Purkinje neurons and stabilise Purkinje resting membrane potential. As such it was postulated that KBr may be effective at reducing the depolarisation blockade caused by lolitrem B within Purkinje neurons, facilitating efferent outputs and therefore reducing the clinical signs of PRGT in livestock.

Currently no effective treatment exists for PRGT in livestock, although a number of approaches have been evaluated to mitigate the clinical signs of PRGT. There have been some indications of efficacy for mycotoxin binding agents (Reed et al. 2011), but 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 varieties of perennial ryegrass is a viable option for disease mitigation however pasture renovation is not suitable or economically viable in many areas of Australia and New Zealand. A viable therapeutic that could be delivered at a flock-wide level would be an important addition to the toolbox of mitigation strategies for this disease. Therefore
the aim of this study was to assess the use of KBr as a suitable therapeutic intervention for PRGT in lambs fed ryegrass seed containing lolitrem B.
7.2 MATERIALS AND METHODS

This study was undertaken with approval from Charles Sturt University Animal Care and Ethics Committee (Wagga Wagga, NSW, Australia).

7.2.1 Animals

White Sussex x Merino first cross male lambs aged 10-12 months (n=43) a live bodyweight (LBW) of 36.1 (SD 3.02) kg were sourced from a single producer. The selection, housing and trial induction of the lambs was as previously described by Combs et al. (2018). At entry to the trial, all lambs were subjected to a full clinical and neurological examination.

7.2.2 Treatments

Lambs were randomly allocated to five treatment groups: Group 1, negative control (n=8); Group 2, positive control (n=8); Group 3, acute KBr treatment (n=9); Group 4, KBr prophylaxis (n=9); Group 5; KBr prophylaxis control (n=9) (Table 7-1). Lambs entered the trial in eight cohorts of five with each cohort containing one animal from each treatment group. A ninth cohort contained Groups 3, 4 and 5 only.

Table 7-1: Description of treatment groups for lambs included in a study to assess the use of potassium bromide (KBr) in sheep being fed endophyte infested ryegrass seed containing lolitrem B.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Feed composition</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>Lucerne chaff</td>
<td>None.</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>Lucerne chaff + ryegrass seed containing lolitrem B</td>
<td>None.</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>Lucerne chaff + ryegrass seed containing lolitrem B</td>
<td>A single dose of 300 mg/kg KBr given after lambs developed neurological signs perennial ryegrass toxicosis.</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>Lucerne chaff + ryegrass seed containing lolitrem B</td>
<td>A loading dose of 540 mg/kg KBr given over 3 days at start of trial, then a dose of 20 mg/kg/day given until end of trial at 21 days.</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>Lucerne chaff only</td>
<td>A loading dose of 540 mg/kg KBr given over 3 days at start of trial, then a dose of 20 mg/kg/day given until end of trial at 21 days.</td>
</tr>
</tbody>
</table>
Chapter 7  Treatment with Potassium Bromide Mitigates Ataxia and Reduces Tremor in Lambs with Perennial Ryegrass Toxicosis

All animals were fed a restricted daily ration at 2.5% live weight. Lambs in Groups 2, 3 and 4 were fed a novel endophyte-infected perennial ryegrass seed (Ga66 AR98; Grasslanz Technology Ltd, Palmerston North, NZ) containing 11 mg/kg lolitrem B. Initially, to acclimatise animals to this diet, seed was fed to provide a dose of lolitrem B of 0.08 mg/kg LBW/day, which was then increased to 0.16 mg/kg LBW /day after 3 days. The remainder of the diet consisted of lucerne chaff (*Medicago sativa*) and 200 mL of 30% w/v molasses (Sprengers, Haigslea, Australia). Lambs in Groups 1 and 5 received only the lucerne chaff and molasses.

7.2.3 Feeding Phase

The initial phase of the study commenced in January 2014, and was designated the Feeding phase. During this phase lambs from Groups 2, 3 and 4 were fed the seed containing lolitrem B and were observed daily for neurological clinical signs, with gait assessment every third day. The first day of feeding 0.08 mg/kg LBW /day lolitrem B was designated Day 0.

Gait assessment was performed by circling lambs in yards adjacent to the housing facility for 3 minutes. The assessment was recorded on video on each occasion and movement scored according to the criteria in Table 7-2, with a maximum score of 5 in six criteria to provide a composite gait score out of 30 for each lamb.
Table 7-2: Clinical observations made during gait assessment of lambs included in a study to assess the use of potassium bromide in sheep being fed endophyte infested ryegrass seed containing lolitrem B.

<table>
<thead>
<tr>
<th>Score</th>
<th>Gait</th>
<th>Rhythmic myoclonus</th>
<th>Directional movement</th>
<th>Ataxia</th>
<th>Ambulation</th>
<th>Cerebellar fits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>Mild disunited fore/hindlimb coordination with acceleration.</td>
<td>Mild hyperextension/hyperflexion.</td>
<td>Reduced ability to rapidly change direction</td>
<td>Mild wide based gait</td>
<td>Stumbling without falling on rapid movement</td>
<td>None</td>
</tr>
<tr>
<td>3-4</td>
<td>Changes in forelimb/hindlimb coordination such as pacing or bunny hopping, exacerbated by increased speed of gait.</td>
<td>Increasing severity, with rhythmic limb hyperextension. Forelimbs typically more affected.</td>
<td>Moves in abnormal directions (i.e. may sometimes fail to follow direction of other animals in group).</td>
<td>Body rolling, wide based gait</td>
<td>Frequent stumbling without falling</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>Continuous bunny-hopping gait at any pace above walk.</td>
<td>All four limbs demonstrate rhythmic hyperextension along with characteristic arcing of back.</td>
<td>Unable to control or maintain direction at any pace above walk.</td>
<td>Body rolling, wide based gait and frequent limb crossing.</td>
<td>Falling</td>
<td>Seizures</td>
</tr>
</tbody>
</table>

*Higher number in grouping represents an increased severity or frequency.*
When lambs in Groups 2 and 3 were observed during gait assessment to show rhythmic myoclonus and stumbling (dropping on hindlimbs or forelimbs) or falling into lateral recumbency, they were transferred from the Feeding to the Testing phase of the trial. If, after 19 days of receiving 0.08 mg/kg LBW/day lolitrem B, lambs had not shown progression of neurological signs, the seed ration was increased to provide a lolitrem B dose of 0.22 mg/kg LBW until clinical criteria for entering the Testing phase of trial were met.

Lambs in Group 1 entered the Testing phase on the same day as the Group 2 lambs in their cohort. Lambs in Groups 4 and 5 remained in the study until Feeding phase Day 18 and then entered the Testing phase.

7.2.4 Testing Phase

On Day 0 of the Testing phase a full clinical examination, with gait assessment and surface electromyography (EMG), as described below, was carried out. On Day 1, a full clinical examination and further gait assessment were conducted. On Day 2, a full clinical examination, gait assessment and EMG were carried out before euthanasia and necropsy. Lambs were injected I/V with 1,000 IU heparin (Wasserberger Arzneimittelwerk, Wasserberg, Germany), followed by 163 mg/kg LBW pentobarbitone sodium (Virbac, Sydney, Australia).

7.2.5 Treatment with KBr

To generate an oral formulation of KBr, 448 g of KBr (Sigma-Aldrich, Sydney, NSW, Australia) was dissolved in 1 L of distilled water to provide a solution containing 300 mg/mL of bromide. All dosages were calculated using bromide ion only.

The anticonvulsant range of concentrations of bromide in serum in monogastric species ranges between 800–2,000 µg/mL (Podell and Fenner 1993). Optimal concentrations that would prevent, attenuate or abolish PRGT tremor in sheep are currently unknown. As such, a dose equating with the mid to lower bound of the monogastric anticonvulsant range was initially used as the target concentration for lambs in Groups 4 and 5. An orally administered loading dose was calculated from the terminal volume of distribution (0.393 L/kg) multiplied by the target concentration (1250 µg/mL) of bromide and an oral
bioavailability of 92% (Quast et al. 2015a). Thus a loading dose of 540 mg/kg bromide PO was required. For lambs in Groups 4 and 5, this was administered over three days; on Feeding phase Day 0, the dose was 300 mg/kg, then 120 mg/kg was administered on the subsequent two days. A maintenance dose of 20 mg/kg/day bromide was then given until the end of the trial. For lambs in Group 3 a single dose of 300 mg/kg bromide was administered on Testing phase Day 0, immediately after completion of gait analysis and surface EMG.

7.2.6 Electromyography

Electrical activity of the triceps muscle was recorded using surface EMG and calculation of root-mean-square (RMS) voltages as previously described (Combs et al. 2018). EMG was undertaken using hydrogel surface electrodes (Kendall, Covidien, MA, USA), placed 20 mm apart. Standing muscle activity was recorded for a minimum of 3 minutes using a bioamplifier, (PowerLab, ADInstruments Dunedin, NZ). Voltages were recorded at a sample rate of 400/second and analysed using LabChart software (ADInstruments). A band pass filter was applied at 20-180 Hz and RMS voltages calculated from a 2 minute epoch.

7.2.7 Measurement of Bromide in Serum and Cerebrospinal Fluid

Blood samples were collected by jugular venepuncture from three Group 3 lambs 6 and 24 hours after treatment on Day 0 of the Testing phase. At necropsy blood was collected from all lambs in Groups 3, 4 and 5. Each blood sample was left to stand for 30 min before centrifugation at 2000g for 5 min followed by harvesting of serum supernatant. Cerebrospinal fluid (CSF) was collected at necropsy from the cisterna pontis via the ventral articulation of the first cervical vertebra.

Concentrations of bromide in serum and CSF were determined by colorimetric spectrophotometry as previously described (Quast et al. 2015). Briefly, 0.5 mL of serum or CSF was added to 4.5 mL of 10% trichloroacetic acid (Sigma-Aldrich) in a 10 mL centrifuge tube, vortexed, then centrifuged for 15 minutes at 2,000g. Then 2.5 mL of supernatant was mixed with 0.25 mL of 0.5% Au₃Cl₆ (Sigma-Aldrich) and left to stand for 30 minutes. Absorbance was measured with an ultraviolet spectrophotometer (Helios λ, Thermo Fisher, Waltham, MA, USA) at 440 nm. The standard curve was linear in the
range of 25 μg/mL to 5,000 μg/mL, $R^2 = 0.9930$. The lower limit of quantification was 25 μg/mL.

### 7.2.8 Concentrations of Cortisol Metabolites in Faeces

Faecal samples were collected from the descending colon of sheep at time of necropsy. Faecal samples were lyophilised, pulverised and homogenised, before a 0.2 g aliquot was taken for metabolite extraction as previously described (Brown et al. 1994). Concentrations of faecal cortisol metabolites (FCM) were measured using ELISA, as described by Narayan et al. (2013) using anti-cortisol polyclonal antibody (R4866, University of California-Davis, CA, USA). Plates were read at 450 nm using an ELx800 (BioTek, Winooski, USA) microplate reader. All FCM concentrations were expressed on a dry faeces weight basis (ng/g). Assay sensitivity, calculated by adding 2 SD to the mean optical density of zero standards, was 28 pg/mL.

### 7.2.9 Histopathology

Full necropsy examination including perfusion fixation of the brain was performed as described previously (Combs et al. 2018). Sections of brain and spinal cord were cut at 5 μm and stained with H&E. The cerebellum was sampled twice, with one longitudinal full thickness mid-sagittal section, and a second sagittal section through the middle of the right cerebellar hemisphere. All sections were assessed for neuropathology and additionally both cerebellar sections were assessed for presence or absence of proximal axonal Purkinje neuron spheroids, as the primary indicator of changes consistent with PRGT. Histopathological examination, reporting of anatomical changes at necropsy and histopathological scoring was undertaken blinded to treatment group by AK and SR.

### 7.2.10 Statistical Analyses

Mixed repeated measures ANOVA was used to assess differences in composite gait score between Groups 1, 2 and 3 on different days. Pairwise comparisons were conducted using Tukey’s adjustment for multiple comparisons, with α set at 0.05. The proportion of lambs falling during gait assessment in the Testing phase was compared between Groups 2 and 3 using Fisher’s exact test.

Mixed repeated measures ANOVA was used to assess differences in RMS between Groups 1, 2 and 3 on different days (Feeding phase Day 0 and Testing phase Days 0 and
2) for. Assumptions of equal variance and normality were violated so log$_{10}$ RMS voltages were used for analyses. Pairwise comparisons were conducted based on estimated marginal means with Bonferroni adjustment for multiple comparisons. RMS voltages were compared between Groups 1, 4 and 5 using a Kruskal-Wallis H test on Feeding phase Day 0 and Testing phase Day 2.

Concentrations of bromide in serum and CSF, and concentrations of FCM at necropsy, were compared between Groups using one-way ANOVA with Tukey’s honestly significant difference test, where appropriate, with $\alpha$ set at 0.05.

The proportion of lambs with granular layer spheroids present in the cerebellum detected on histopathological examination was compared between Groups using logistic regression analysis.

Analyses were undertaken using IBM SPSS version 20.0.0 (IBM, Armonk, NY, USA), and Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

7.3 RESULTS

One animal in Group 2 was not included in the analyses as it was not correctly identified throughout the study.

7.3.1 Neurological Observations and Gait Assessment

There was considerable variation in the observed onset of clinical signs between lambs that were fed seed containing lolitrem B. The median interval from the start of the Feeding phase to first clinical signs for lambs in Group 2 was 7 (min 3, max 7) days, in Group 3 was 9 (min 6, max 12) days and in Group 4 was 6 (min 3, max 9) days. By Day 19 of the Feeding phase 5/7 lambs in Group 2 and 7/9 lambs in Group 3 had not met the clinical criteria for entering the Testing phase and so were fed the maximal dose of 0.22 mg/kg LBW/day lolitrem B. Lambs in Group 2 were given this dose for between 4–14 days and lambs in Group 3 for between 5–20 days.

Median interval to onset of first detectable gait changes for Group 2 was 8 (min 6, max 20) days, for Group 3, 9 (min 8, max 15) days and Group 4, 12 (min 7, max 20) days.

The median interval from the start of the Feeding phase to meeting the criteria for entry to the Testing phase for lambs in Group 2 was 24 (min 17, max 33) days, and for lambs
in Group 3 was 29.5 (min 17, max 39) days. All lambs in Groups 4 and 5 entered the Testing phase on Day 18.

The composite gait score for lambs in Groups 1 and 5 did not change during the Testing phase (Table 7-3). The repeated measures analysis of composite gait scores for lambs in Groups 1, 2 and 3 showed a significant interaction between Days and Groups (p<0.001), as well as a main effect of Group (p<0.001), indicating that there was a difference in the way the Groups responded on different days. The mean composite gait scores of lambs in Groups 2 and 3 were similar on Testing Day 0 (p=0.09). In Group 3 lambs, mean scores decreased between Testing phase Day 0 and Days 1 and 2 (p<0.001), whereas mean scores increased in lambs in Group 2 between Day 0 and Day 2 (p=0.015, Table 7-3). On Testing phase Day 2 lambs in Group 3 had lower mean scores compared to lambs in Group 2 (p<0.001).

The proportion of lambs falling during gait assessment was less following KBr treatment in Group 3 lambs than in untreated Group 2 lambs on Testing phase Days 1 (p=0.011) and 2 (p=0.003) (Table 7-3).

In lambs in Group 4, the first neurological signs were observed on average 6.33 (SD 1.73) days after the start of the Feeding phase, and included positional ventral strabismus in one lamb, a fine tremor of the head and neck in two lambs, lethargic demeanor in three lambs, mild truncal ataxia at rest in one lambs or mild wide base stance in three lambs. In Group 5 lambs, a mildly lethargic demeanor in four lambs and wide based stance in four lambs was also observed on average 10.75 (SD 4.64) days) after the start of the Feeding phase. Lambs in Groups 4 and 5 exited the study 21 days after the start of the Feeding phase thus precluding the analysis of more significant neurological signs.
Table 7-3: Mean (±SD) composite gait scores and proportion of animals falling during gait assessment in the Testing phase of a trial to assess the effect of potassium bromide (KBr) in lambs being fed endophyte infested ryegrass seed containing lolitrem B.

<table>
<thead>
<tr>
<th>Treatment group a</th>
<th>Group 1 (n=8)</th>
<th>Group 2 (n=7)</th>
<th>Group 3 (n=9)</th>
<th>Group 4 (n=9)</th>
<th>Group 5 (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gait score</td>
<td>8</td>
<td>7</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Testing Phase Day 0</td>
<td>0.0±0.0</td>
<td>14.8±3.0 s</td>
<td>19.14±2.2 s</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Testing Phase Day 1</td>
<td>0.0±0.0</td>
<td>19.0±2.9 t</td>
<td>10.0±5.4 t</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Testing Phase Day 2</td>
<td>0.0±0.0</td>
<td>21.7±3.3 rw</td>
<td>8.8±4.8 tz</td>
<td>8.4±3.9</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>No. falling (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testing Phase Day 0</td>
<td>0 (0)</td>
<td>7 (100)</td>
<td>9 (100)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Testing Phase Day 1</td>
<td>0 (0)</td>
<td>7 (100) y</td>
<td>3 (33) z</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Testing Phase Day 2</td>
<td>0 (0)</td>
<td>7 (100) y</td>
<td>2 (22) z</td>
<td>1 (11)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

a Group 1 fed lucerne chaff; Group 2 fed ryegrass seed containing lolitrem B; Group 3 fed ryegrass seed containing lolitrem B and treated with KBr after showing clinical signs; Group 4 fed ryegrass seed containing lolitrem B and treated with KBr from the start of the trial; Group 5 fed lucerne chaff and treated with KBr from the start of the trial

s, t Mean scores differ between Days (p<0.05)

w, x Mean scores differ between Groups (p<0.001)
y, z Proportions differ between Groups (p<0.05)

7.3.2 Electromyography

The repeated measures analysis of RMS voltages for lambs in Groups 1, 2 and 3 showed a significant interaction between Days and Groups (p=0.017), as well as main effects of Day (p<0.001) and Group (p=0.003), indicating that there was a difference in the way the Groups responded on different days.

For lambs in Group 2 mean RMS voltage was increased on Testing phase Days 0 (p=0.01) and 2 (p=0.001), compared with Feeding phase Day 0. Similarly, for lambs in Group 3, mean RMS voltages was increased on Testing phase Day 0 (p=0.003) and 2 (p=0.026) compared with Feeding phase Day 0. There was no difference in mean RMS voltages
measured on Feeding phase Day 0 and Testing phase Days for lambs in Group 1
(p=0.307) (Figure 7-1).

On Testing Phase Day 2 mean RMS voltages were higher in Group 2 compared with
Group 1 (p=0.001) and Group 3 (p=0.045) lambs. Mean RMS voltages were similar in
lambs from Group 1 and Group 3 on Day 2 (p=0.117) (Figure 7-1).

The distribution of RMS voltages measured in lambs from Groups 1, 4 and 5 on Feeding
phase Day 1 and Testing phase Day 2 did not differ between Groups, as determined using
the Kruskal-Wallis H test ($\chi^2 = 3.772, p = 0.152$). On Testing phase Day 2 median RMS
voltages were as follow: Group 1, 0.021 (min 0.013, max 0.037) mV, Group 4 0.039 (min
0.012, max 0.199) mV, Group 5, 0.018 (min 0.009, max 0.028).

![Figure 7-1](image)

Figure 7-1. Geometric mean (with 95% CI) root mean square voltages of surface
electromyograms recorded from the triceps muscle of lambs fed lucerne chaff
(white bars), or ryegrass seed containing lolitrem B (light grey bars), or ryegrass
seed containing lolitrem B and treated with potassium bromide after showing
clinical neurological signs (dark grey bars). Recordings were made at the start
of feeding (Feeding Trial Day 0) and on Days 0 and 2 of the Testing Phase. *
Treatment groups differed (p=0.045).

7.3.3 Concentrations of Bromide in Serum and CSF
Mean concentrations of bromide in serum measured in lambs from Group 3 after
administration of a single dose of 300mg/kg KBr were 899.4 (SD 97.8)$\mu$g/mL (n=3) at 6
hours, 868.6 (SD 78.9) µg/mL (n=3) at 24 hours, and 804.3 (SD 59.4) µg/mL (n=6) after 48 hours.

Mean concentrations of bromide measured in serum and CSF of lambs in Groups 3, 4 and 5 after euthanasia on Day 2 of the Testing phase are shown in Table 7-4. Concentrations were higher in samples from lambs in Group 4 than Group 5 in both serum (p=0.011) and CSF (p=0.008).

**Table 7-4: Mean (±SD) concentrations of bromide (µg/mL) in serum and cerebrospinal fluid (CSF) measured following euthanasia on Day 2 of the Testing phase of a trial to assess the effect of potassium bromide (KBr) in lambs being fed endophyte infested ryegrass seed containing lolitrem B.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Concentration (µg/mL)</th>
<th>CSF Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>804.3±59.4</td>
<td>635.1±128.8</td>
</tr>
</tbody>
</table>
| 4       | 830.1±114.8
         | 714.5±141.2               |
| 5       | 651.9±105.7                | 459.3±115.8               |

a Group 3 fed ryegrass seed containing lolitrem B and treated with KBr after showing clinical signs; Group 4 fed ryegrass seed containing lolitrem B and treated with KBr from the start of the trial; Group 5 fed lucerne chaff and treated with KBr from the start of the trial.
yz Mean scores differ between Groups (p<0.05)

7.3.4 Concentrations of Cortisol Metabolites in Faeces

Mean concentrations of FCM measured at necropsy differed between treatment groups (p=0.038; Figure 7-2). Concentrations were lower in samples from lambs in Group 1 than Group 2 (p=0.02) and were lower in samples from lambs in Group 5 than Group 2 (p=0.012), but were similar in lambs from Groups 2, 3 and 4 (p>0.05).
Chapter 7  Treatment with Potassium Bromide Mitigates Ataxia and Reduces Tremor in Lambs with Perennial Ryegrass Toxicosis

Figure 7-2: Mean (± SEM) concentrations of cortisol metabolites (FCM) in faeces collected at necropsy from lambs fed lucerne chaff (Group 1), or ryegrass seed containing lolitrem B (Group 2), or ryegrass seed containing lolitrem B and treated with potassium bromide (KBr) after showing clinical neurological signs (Group 3), or fed ryegrass seed containing lolitrem B and treated with KBr from the start of the trial (Group 4) or fed lucerne chaff and treated with KBr from the start of the trial (Group 5). * Treatment groups differ (p<0.05).

7.3.5 Histopathology

Sections from all cortical and subcortical regions were examined, but neurohistopathological lesions identified were restricted to sections from the cerebellum. Sections from lambs in Groups 2, 3 and 4 showed similar neurohistopathological changes; namely pyknosis of neurons within the granular (innermost) layer of the cerebellum, Purkinje neuron vacuolation and Purkinje neuron proximal axonal spheroid formation. The proportion of lambs exhibiting Purkinje neuron proximal axonal spheroids were 0/8 in Group 1, 6/7 in Group 2, 5/9 in Group 3, 6/9 in Group 4 and 1/9 in Group 5. No lambs in Group 1 exhibited spheroids. Compared with Group 2, the odds of having spheroids were 98 (95% CI=88–100) % less in Group 5 (p< 0.001), but the odds were similar in lambs from Groups 3 and 4 (p>0.05). Spheroids were noted in the molecular (outer) layer
in 3/9 lambs from Group 4, 3/9 lambs in Group 5 and 1/8 lambs in Group 1, but none in Groups 2 or 3.

7.4 DISCUSSION

In this study lambs fed ryegrass seed containing lolitrem B, with moderate tremor and gait abnormalities, that were treated with a single oral dose of KBr at 300 mg/kg had reduced composite gait scores (as a measure of gait abnormalities) and a reduced likelihood of falling compared with comparable lambs not treated with KBr, indicative of mitigation of the neurotoxic effects of lolitrem B. Lambs treated with KBR also had a reduction in RMS voltages from surface EMG of the triceps muscle, also indicative of mitigation of tremor and ataxia. These results suggest a significant improvement in clinical signs of ataxia, movement disorder and muscle tremor associated with PRGT in sheep treated with oral KBr.

In this study, lambs fed ryegrass seed containing lolitrem B were administered a single oral dose of KBr (Group 3) when exhibiting clinical signs typical of those found with naturally occurring PRGT. The ability of KBr as an oral treatment to reduce tremor, improve neurological signs and normalise ambulation could have a profound effect on a farmer’s ability undertake husbandry procedures on affected stock, to move stock in cases of severe outbreaks and also to treat recumbent animals.

As found in naturally occurring field cases (Combs et al. 2014), and previous controlled trials (Combs et al. 2018a), histological lesions identified in lambs fed ryegrass seed containing lolitrem B in this study were restricted to the cerebellum. The classical lesions associated with PRGT were noted, namely Purkinje neuron vacuolation and Purkinje neuron proximal axonal spheroid formation, along with granular layer neuron pyknosis (Combs et al. 2018). Purkinje neuron loss observed in this study could be due to cerebellar neuronal degeneration arising as an adaptive response to membrane depolarisation, resulting from BK channel blockade after exposure to lolitrem B toxin. In mice, Purkinje neuron atrophy was found to be an adaptive response to Purkinje neuron membrane destabilisation induce by a reduction in BK channels (Dell'Orco et al. 2015). Our findings have suggested that neuronal cell loss within the granular layer could be an early histopathological lesion indicative of increased membrane instability in the cerebellum, in animals suffering from PRGT (Combs et al. 2018). However the mechanism
responsible for neuronal degeneration observed in PRGT-affected animals is unknown and further research is necessary to determine the mechanism of neuronal cell loss.

Treatment with KBr did not result in any differences in the underlying neurohistopathological lesions associated with induced PRGT in this study; the proportion of lambs with spheroid lesions did not differ between any of the groups fed ryegrass seed containing lolitrem B, regardless of treatment. The activity of KBr is not likely to be a specific antagonist of lolitrem B, but rather a non-specific mitigator of clinical signs, therefore it may have been unrealistic to expect that the histopathological features of PRGT, such as the formation of axonal spheroids in the cerebellum, would be absent in treated animals. Additionally, in Group 3 lambs, assessment of histopathological lesions occurred only 48 hours after treatment with KBr commenced and histopathological differences may require longer to become apparent. A detailed morphometric analysis should also be considered to determine the numbers of e.g. axonal spheroids in future studies.

Concentrations of FCM measured postmortem were also not statistically different between the groups fed ryegrass seed containing lolitrem B (Figure 7-2). However only lambs fed this seed that were untreated (Group 2) had significantly increased concentrations of FCM when compared to negative control lambs. In both groups treated with KBr, concentrations of FCM were similar to those of negative control lambs. Whether treatment with KBr is able to mitigate the stress response observed in animals with PRGT as well as mitigating their movement disorder warrants further investigation, particularly in the light of the important implications such a response would have for the improved ethical management of PRGT-affected flocks.

A cost-effective prophylactic therapy for PRGT would be highly desirable for farmers with ryegrass dominant pastures. In this study prophylactic administration of oral KBr (Group 4) delayed the onset of significant gait changes in lambs fed ryegrass seed containing lolitrem B. However lambs treated with a daily prophylactic dose exhibited more variable changes in clinical presentation than those lambs treated with a single acute dose of KBr. One limitation in this study was the endpoint for the prophylactic treatment groups; this was set at 18 days after the start of the feeding phase, which was anticipated to be twice the time required for lambs to meet the clinical criteria for entry into the Testing phase of the study. However progression to these clinical signs was found to be
highly variable, with the median interval being >21 days for lambs in Groups 2 and 3. Another feature of the lambs in Group 4 was that some of them showed early neurological changes. The reasons for this are unclear but it is possible that the loading dose of KBr may have been too high, as early clinical signs were typical of animals with bromide-related sedation (Podell and Fenner 1993; March et al. 2002), which therefore presented a confounder in the prophylaxis component of this study. Despite this, KBr may potentially be useful as a prophylactic treatment for PRGT, but further studies are needed to determine an effective regimen in sheep.

Concentrations of bromide in serum reached approximately the lower end of the therapeutic range (800 to 2000 µg/mL) reported in monogastric species (Podell and Fenner 1993). Upper and lower limits of efficacy of KBr in sheep have not yet been defined, however in this study KBr was well tolerated. The positive results in this study suggest that KBr has good efficacy for treatment of PRGT after administration of a single dose of 300mg/kg, although animals in naturally occurring disease outbreak can be severely affected and higher dosing may be required in some animals.

Although sedative effects were observed in lambs treated prophylactically with KBr, no observations of clinically significant neuropathies or other signs of toxicity (bromism) were noted. Signs of bromism in non-ruminant species include gastric irritation, stupor, ataxia, tremor, skin rashes, respiratory signs and hindlimb paresis (Nuki et al. 1966; Boothe 1998; Bertolani et al. 2012). Despite the apparent safety of KBr in sheep, further studies should be undertaken to quantify the safety profile of KBr for use on commercial flocks.

Concentrations of bromide in CSF of lambs treated with KBr were similar to those previously reported in dogs (March et al. 2002), however lambs in Group 4 had increased concentrations compared to those in Group 5. If bromide is selectively retained in the CNS of animals fed seed containing lolitrem B it would suggest a lower prophylactic dose of KBr would be sufficient to achieve the therapeutic concentrations required in the central nervous system.

Further assessment of the efficacy of KBr for on-farm treatment of PRGT is required to develop practical protocols for use in the field. Nevertheless, the marked improvements
in gait observed in this study and the reduction in tremor without sedation, on delivery of a single oral dose of KBr are important characteristics of this therapy.

7.5 ACKNOWLEDGEMENTS

The authors wish to thank Cate Chandler, Alice Birckhead, Emily Birckhead, the staff of the Veterinary Teaching Hospital and the Veterinary Diagnostic Laboratory, Charles Sturt University, for their excellent technical assistance during this study. Thanks to Adam Hamlin for assistance with perfusion fixations. JC Quinn was supported by funding from Meat and Livestock Australia and a Research Fellowship from the Graham Centre for Agricultural Innovation (Charles Sturt University and NSW Department of Primary Industries). MD Combs was supported by a technical assistance grant from Meat and Livestock Australia.

Additional material relating to this chapter can be found in Appendix 10.5 and 10.6.
Chapter 8  DISCUSSION

8.1  SCOPE OF STUDY

The set of research studies encapsulated in this PhD is built around a central question: Could bromide be a practical, on-farm, therapeutic agent for ameliorating PRGT? To answer this, a series of research questions arose and suitable experimental models for PRGT in which testing could occur needed to be developed. In this PhD these studies have been arranged into chapters with each chapter focusing on a piece of the larger puzzle.

Chapter 2 is a review of the development of knowledge regarding PRGT, its clinical expression, pathogenesis and the experimental techniques used in PRGT research. Other fields of neuroscience that are of relevance to techniques employed within this study are also discussed.

The third chapter seeks to characterise the clinical expression of PRGT in southern Australia through the examination of a specific PRGT outbreak in south western Victoria. Clinical signs, including neurological signs, are recorded along-side clinical pathology, histopathology and necropsy findings. These animal findings are then put in the context of the clinical history, local farming systems and pasture analysis findings. Key features of this study are the detailed histopathological findings and examination of clinical pathology results from field cases both of which improved understanding of the pathogenesis of PRGT.

The fourth chapter is a study of the clinical expression of lolitrem B and paxilline intoxication in a mouse model. This chapter investigates a number of novel testing modalities (parallel rod, piezoelectric pressure sensor and Barnes maze), used to improve the understanding of the neurological changes expressed with PRGT. Key findings in this chapter are the demonstration of spatial orientation deficits, inhibition of voluntary movement and the development of a new technique to quantify the tremorgenic effects of lolitrem B and paxilline (the piezoelectric pressure sensor and the use of a tremor-motion power ratio).
In the fifth chapter bromide, identified as the primary therapeutic agent, is tested. Refinement of experimental techniques and knowledge gained from the fourth chapter is applied to assess the response to bromide therapy in lolitrem B intoxicated mice. c-Fos immunoreactivity in the forebrain is also compared between bromide pre-treated animals and controls. This chapter clearly demonstrates that bromide pre-treated mice have a reduced tremor, an increased propensity to voluntary motor activity and a reduced perception of stress following lolitrem B intoxication.

The sixth chapter looks at the expression of PRGT in sheep, using a pen fed laboratory model. Within this model intensive clinical and neurological observations are made allowing the development of a composite gait score to define changes in movement. Also a novel method of surface EMG is demonstrated to be effective at recording and scaling intensity of tremor/ataxia in PRGT affected sheep. Increased levels of faecal cortisol metabolites and water intake deficits are also identified.

The seventh chapter uses the laboratory based disease model developed in Chapter 6 to test the efficacy of bromide therapy for PRGT. This chapter demonstrates that orally administered bromide produces little to no sedation (unlike other many anti-tremor agents) and yet is effective at reducing EMG recorded tremor activity and cerebellar/vestibular ataxic effects of lolitrem B intoxication, thus greatly reducing the propensity to falling.

### 8.2 Advancements in the Clinical/Laboratory Assessment of PRGT

The research contained in this thesis demonstrates a number of important advancements in the assessment of the complex toxicity that is PRGT.

The assessment of naturally occurring clinical cases, in Chapter 2, demonstrated the variation in environmental conditions under which PRGT can occur and that dehydration may play a critical role in progression of the toxicity in naturally occurring disease outbreaks. My investigations also developed a set clinical criteria that has important implications for the assessment of animals in the field. Previous to this work the Keogh scale involved the driving of affected animals to determine the extent of their dysfunction but with the inherent risks of injury and/ or inability to rise from falling of large numbers of animals. The scale elaborated in this study was a simplified clinically relevant scale (Type 1 or Type 2), based on observation only, without significant intervention. These
criteria sought to highlight the difference between gait abnormalities that are unlikely to produce severe disease outcomes (Type 1, dysdiadochokinesia or a disorder of limb sequencing) and gait changes with a high probability of falling/ injury/collapse/ death (Type 2, rhythmic myoclonus). Highlighting the critical differences of gait changes should assist veterinary clinicians and livestock producers in making management decisions regarding affected stock in the future.

In the mouse model studies, reported in Chapters 4 and 5, the demonstration of the use of a piezoelectric pressure sensor to refine tremor observations and to characterise the nature of the tremor and locomotor changes are new findings that allow a more detailed assessment of tremor in disease states. Quantitative data from this assessment tool was used to assess the efficacy of bromide therapy, providing ample evidence of their efficacy and validity for future experimental work on tremorgenic toxins.

Observation of reduced voluntary locomotion in mice following lolitrem B intoxication has not been noted in previous studies and is an important clinical indicator that has critical implications in livestock management, as toxin effects on grazing and water seeking behaviours are likely to be affected. Likewise demonstration of spatial orientation deficits without cognitive deficits from lolitrem B-induced BK channel blockade were important findings with implications for large animal clinical practice. The combination of spatial orientation deficits and activation of neural stress pathways (Chapter 5) would seem a plausible explanation for mass drowning events. Approaching watering points is a stress activator in herbivores, representing a sight of predation (Makin et al. 2017). Lolitrem B intoxication will serve to heighten behavioural alertness and responses to perceived threats but decreased spatial orientation. As such an intoxicated herd would be at increased risk of a flight response but with poor control of direction, hence running into the water body.

Development of a sheep disease model also demonstrated a number of important assessment procedures. The key underlying (and novel) component of the protocol used in this study was the use of discrete clinical entry criteria for testing rather than a set time-point. This was a critical approach as time-to expression of clinical signs, following oral consumption of toxin, is highly variable. A clinical-criterion-based experimental protocol therefore allowed testing of animals at a similar clinical stage.
The use of a composite gait score allowed for improved differentiation of neurological deficits (for example gait versus spatial orientation deficits) and was a useful tool in assessing the degree on neurological dysfunction. It has some advantages over the “Keogh scale” (Keogh 1973) in that the Keogh scale does not provide a detailed gait assessment and groups the progression of different neurological signs in a manner that may not always occur in the intoxicated animal.

Assessment of surface EMG to measure tremor in sheep was an adaption of techniques described by Smith et al. (1997). Surface electrodes were moved from the shoulder, as previously described, to the triceps muscle as this muscle group was observed to be highly active during PRGT intoxication. Another adaption was the use of root-mean-squared analysis of fixed time periods to allow quantitative assessment of the degree of muscle activity. Surface EMG using this technique provides a sensitive quantitative test, useful in assessing degree of intoxication and therapeutic response, and should be widely applicable for measuring effects of other tremorgenic toxins in livestock.

8.3 ADVANCEMENTS IN UNDERSTANDING OF AETIOPATHOGENESIS OF PRGT

To improve the understanding of the pathogenesis of PRGT, perfusion fixation and histopathological assessment of brains from sheep was performed during the controlled sheep model of PRGT. This work confirmed previously disputed observations (Mason 1968) from field cases of PRGT. Identifiable neurological lesions were confined to the cerebellum, with increased proximal axonal bodies and vacuolation present in Purkinje neurones. Additionally, this study identified that spheroids were present in increased numbers in the vestibulocerebellum providing both a key location for these lesions as well as an association with identified clinical signs, suggesting a pathological mechanism. My histopathological studies also identified a previously unreported finding of pyknosis in granular layer neurones. This combination of changes (pyknosis in the granular layer, high numbers of spheroids in the vestibulocerebellum) will improve the ability to specifically diagnose PRGT from histopathological samples of the cerebellum in naturally occurring disease outbreaks.

As in humans with essential tremor, the use of presence of spheroids in the cerebellum as a key diagnostic criteria for PRGT has been disputed because lesions can been found in older animals that do not have PRGT (Mason 1968; Louis et al. 2007). In humans a seven
fold increase in Purkinje neuron proximal axonal bodies were noted compared to controls; counts of spheroids in anatomically identical sections of cerebellum would be a natural progression of the work undertaken in this thesis to aid veterinary pathologists in the diagnosis of this disease in clinical samples.

Histopathology from field cases (Chapter 2) and controlled sheep and rodent studies (Chapter 4 and 5) did not suggest supra-tentorial lesions were present in animals exposed to lolitrem B toxin. To examine the hypothesis that forebrain dysfunction was present with lolitrem B intoxication, an immunohistochemical study using the neuronal activation marker c-Fos was undertaken (Senba et al. 1993). Activation of stress response neural pathways were identified in mice post lolitrem B intoxication. My investigations also identified that bromide reduced activation of the central amygdala, a central processing area for control of the emotional response to stress in humans and animals (van der Kooy et al. 1984; Van de Kar and Blair 1999; Partridge et al. 2016). This suggests that bromide altered the perception of stress in animals exposed to lolitrem B. This finding has important animal welfare implications for PRGT affected animals and has broader implications regarding the potential use of bromide as a stress mitigation strategy in livestock.

In response to findings of a stress response in lolitrem B intoxicated mice (and observations by Johnstone (2011) of allodynia in equine models of PRGT) a number of modalities were employed to investigate stress/pain/allodynia responses with PRGT in sheep (Johnstone 2010). In my studies mechanosensory nociceptive threshold testing was confounded by the impacts of neurological deficits, preventing an assessment of allodynia. However faecal cortisol testing clearly demonstrated an increase in a physiological stress response in intoxicated flocks. As faeces can be collected easily without disturbing flocks and this is a relatively simple quantitative test to perform, it could be of great value if used for monitoring flocks with a known history of PRGT and may be helpful in decision making around timing therapeutic interventions.

8.4 ADVANCEMENTS IN THERAPEUTIC OPTIONS FOR PRGT

The research reported in this thesis clearly demonstrates the potential of bromide as a therapeutic intervention for PRGT. Trials in both sheep and mice demonstrated that treatment with bromide was effective at reducing tremor and improving mobility. The
results of this study has major implications for livestock grazing ryegrass predominant pastures. Field trials should be carried out to determine the best treatment regimens for therapy and investigate potential productivity implications of using bromide as a prophylactic therapy. This assessment should occur prior to any large scale therapy roll-out. The long half-life of bromide (Quast et al. 2015), and hence the potential for accumulation at supra-therapeutic levels, needs to be further investigated in any prophylactic therapeutic regimens. Delivery modality will also be critical as self-administration such as salt licks will not be suitable for on-farm therapy, except at low doses, and individual slow release ruminal devices are likely to be preferable.

8.5 COMPARATIVE ASPECTS OF RESEARCH

Essential tremor is the most common tremor condition in humans. Furthermore, lolitrem B produced both a cerebellar tremor and histopathological lesions similar to those found with essential tremor in humans. The research in this thesis clearly demonstrates the utility of bromide as a therapeutic for cerebellar tremor, which has significant implications for human medicine and the treatment of cerebellar dysfunction in any species and should be further investigated.

Intoxication with lolitrem B also appeared to induce stress, and essential tremor has been found to be exacerbated by stress, although any causal relationship is unclear (Anouti and Koller 1995, Pahwa et al. 2003). These findings highlight the potential for lolitrem B/PRGT to be used as a disease model for essential tremor research.

8.6 OVERALL OUTCOME SUMMARY

PRGT is a complex toxicity with climate, endophyte strain, environment, pasture, farming system and animal factors all affecting the presentation of the disease. Dealing with these complexities likewise needs a multi-factorial approach and before the start of this doctorate I identified the lack of a viable therapeutic as a key factor missing to aid farmers manage this toxicity.

Tackling the issue of a therapeutic could be considered a bold project within a PhD. No laboratory models of PRGT existed that were suitable for drug testing, likewise no potential therapeutic agents had been identified that were suitable for flock-wide on-farm delivery. Therefore a broad project-based approach was taken that utilised both small and large animal models of disease with many novel testing modalities. The novel
findings identified in this thesis regarding the neurological changes in PRGT affected animals provide significant additional insight into this disease. Furthermore the testing modalities developed for this doctorate add to the tools that can be used to further research this toxicity and other tremorgenic/neuroactive toxins.

This doctorate has resulted in the identification of a drug with potential for large scale use against a recurrent pasture intoxication. This is a major breakthrough in the management of tremorgenic intoxications in grazing animals.
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Chapter 10 APPENDICES
10.1 Appendix 1: Data Relating to Chapter 3 Clinical Presentation of Perennial Ryegrass Toxicosis Outbreak in Australia.

10.1.1 Farm Locations Visited During Chapter 3 Study

Figure 10-1: Locations of farms visited during 2011 outbreak of PRGT in South-West Victoria
10.1.2 Pasture Inspection of Renovated Pasture using Novel Endophyte where an Outbreak of PRGT had occurred.

Figure 10-2: A paddock from which animals were removed following demonstrating signs of PRGT. This paddock was sown with a novel endophyte variety of perennial ryegrass (*Endo 5®*) and subclover.

Figure 10-3: Identification of ryegrass varieties in paddock. Close inspection of the paddock reveals that two varieties of ryegrass are present, *Endo 5®* (yellow arrow) and wildtype (red arrow). Subclover growth is sparse only (orange arrow). Invasion of wildtype variety has occurred despite the owner following recommendations on pasture renovation with novel endophyte cultivars.
10.1.3 **HPLC Analysis of Pasture Samples taken during the 2011 PRGT Outbreak in SW Victoria.**

*Table 10-1: HPLC analysis of pasture samples taken during the 2011 PRGT outbreak in SW Victoria*
Chapter 10.1  Appendix 1: Data Relating to Chapter 3 Clinical Presentation of Perennial Ryegrass Toxicosis Outbreak in Australia

<table>
<thead>
<tr>
<th>Date</th>
<th>Case</th>
<th>Symptoms</th>
<th>Treatment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>11/11/11</td>
<td>01</td>
<td>Mild</td>
<td>Treatment A</td>
<td>Recovered</td>
</tr>
<tr>
<td>12/11/11</td>
<td>02</td>
<td>Moderate</td>
<td>Treatment B</td>
<td>Recovered</td>
</tr>
<tr>
<td>13/11/11</td>
<td>03</td>
<td>Severe</td>
<td>Treatment C</td>
<td>Recovered</td>
</tr>
<tr>
<td>14/11/11</td>
<td>04</td>
<td>Critical</td>
<td>Treatment D</td>
<td>Died</td>
</tr>
</tbody>
</table>

Note: All treatments were administered within 24 hours of symptom onset.
10.1.4 Examples of Clinical Cases Observed in Hamilton Victoria Region during 2011 PRGT Outbreak.

Figure 10-4: A merino lamb that fell into lateral recumbency during moving stock. This animal had displayed type 2 gait changes with rhythmic hyperextension of limbs. The animal is good condition and does not have scours (note normal faeces). The animal did regain its feet after a short period but had poor directional movement, struggling to rejoin the flock. The large amount of feed was unusual in this region.
Figure 10-5: Sheep A1: An animal that has been in prolong recumbency (note the wear patch on pasture and the emaciated condition of the animal). This ewe was part of a flock of 1450 composite ewes. Of the flock, the farmer had estimated 250 had gone down, 15 had already died in recumbency and 43 had drowned in a dam. The farmer was struggling to maintain adequate treatment and welfare for this flock.

Figure 10-6: Sheep D10. This animal has fallen near a fence and in its struggles to stand has become trapped under a fence. As such, this animal might typically be classified as a “death by misadventure”. Markings on the ground suggest this animal may have been in this situation for some time and elevation of sodium on clinical pathology is consistent with the water deprivation you might expect in this animal. On clinical examination, this animal exhibited extension of the neck, strabismus, fine tremor and severe motor incoordination. Mild enophthalmos was the only clinical indicator of dehydration. The extent of neurological changes led this animal to be euthanased.
Figure 10-7: Several other animals on the same property remained in sternal recumbency. The large amount feed meant that these animals remained in good condition despite being unable to stand for several days.
10.1.5 Histopathological Sections of Cerebellum Demonstrating Axonal Swelling at Varying Lesion Density.

Figure 10-8: Sheep D6 (top) and D8 (below): Histological section (H&E) of the cerebellum. Both these animals also show axonal swellings of Purkinje neurons within the granular layer. These are particularly prominent in D8, although they are not in the classical torpedo shape. Note also the presence of pyknotic granule cell neurons (small dark nuclei in the granular layer), indicating granule cell apoptosis. Given the fixation process this could represent post-mortem changes and so this is investigated further in Chapter 6 where perfusion fixation of brains allowed for more detailed observations.
10.2 APPENDIX 2: DATA RELATING TO CHAPTER 4: CHARACTERISATION OF NEUROLOGICAL DYSFUNCTION INDUCED BY THE BK CHANNEL ANTAGONISTS PAXILLINE AND LOLITREM B USING A MOUSE MODEL.

10.2.1 Preliminary Study: Identification of Tremor Spectra and Refinement of Tremor Sensor

Figure 10-9: A Composite fast Fourier transform (FFT) graph of power-frequency spectrum at all time-points within the tremor characterisation study (preliminary data not within the chapter). The largest tremor peak for lolitrem B intoxicated mice (A) was at 3 hours, whereas in Paxilline intoxicated mice (B) it was at 1:30 hours. The selected tremor frequency range is indicated (vertical lines, 15-25Hz). Tremor-Movement Power ratio is then calculated by calculation area under the curve (AUC) for FFT spectrum within the tremor frequency range, expressed as a ratio to AUC for FTT spectrum from 0-50 Hz. The absence of power output in frequencies 0-10Hz (frequencies associated with normal movement) indicated the device design was absorbing low frequency movement. The design was corrected and more normal spectrum were evident in later studies (Chapters 4 and 5).
10.2.2 Preliminary Study Data: Tremor-Motion Power Ratio in Lolitrem B intoxicated mice.

Figure 10-10: Tremor-Motion Power Ratio (TMPR) across the 72 hour testing period (time-points not plotted to scale) in lolitrem B intoxicated mice (n=4).

10.2.3 Preliminary Study Data: Tremor-Motion Power Ratio in Paxilline intoxicated mice.

Figure 10-11: Paxilline Tremor-Motion Power Ratio (TMPR) across 5 hours, (n=4)
10.2.4 Preliminary Study Data: Movement on Parallel Rod Device.

Figure 10-12: Effect of paxilline (6mg/kg IP, n=4) and lolitrem B (2mg/kg IP, n=4) on distance travelled (meter, m) by parallel rod test. Exposure to both lolitrem B and paxilline significantly reduced the distance travelled (P<0.0001) when compared to pre-treatment values. Paxilline treated animals showed significantly decreased movement between 30 minutes and 3 hours post intoxication (*, P≤ 0.05). Lolitrem B treated animals showed significantly decreased distance travelled between 2 hours and 8 hours post intoxication (**, P≤0.01)
10.2.5 **Composite FFT from Piezoelectric Pressure Sensor in Paxilline Intoxicated Mice.**

![Composite FFT for Paxilline intoxicated mice](image)

**Figure 10-13:** Composite FFT from piezoelectric pressure sensor in paxilline intoxicated mice (n=7) on each day of behavioural trial
10.2.6 Composite FFT from Piezoelectric Pressure Sensor in Mice following 90% DMSO (vehicle) via IP Injection

Figure 10-14: Composite FFT from piezoelectric pressure sensor in mice receiving a 90% DMSO injected mice (n=7) on each day of behavioural trial.
10.2.7 Video Capture from a Novel Object Recognition Test showing Tracking Software View.

Figure 10-15: Video Capture from novel object recognition (NOR) test. Two objects (black ball and white cube). White cube is indistinct due to high contrast filming technique used) sit equidistant from their nearest corner. The head of the mouse entering the outer ring was used to test the animal’s interaction with the two objects.
10.2.8 **Novel Object Recognition: Heat Maps from Head Tracking**

Figure 10-16: Examples of head tracking heat map plots at 2 (A, E), 26 (B, F), 50 (C, G) and 74 (D, H) hours post lolitrem B 2mg/kg IP (A-D) or no treatment control (E-H). (A-D) At 2 hours lolitrem B intoxicated mouse show little exploration, at 26 hours exploration behaviour is increased however mice rarely crossed the centre of experimental field. By day 3 and 4 exploration behaviours appear more normal. (E-H) Contrary to lolitrem B intoxicated mouse, control mouse extensively explores both objects and arena from 2 hours.
10.2.9 Parallel Rod Apparatus

Figure 10-17: (A) ANY-maze™ (Stoelting Co., Wood Dale, IL, USA) Parallel Rod Apparatus. (B) This image demonstrates the view taken by digital video capture. During testing a high contrast setting appeared to aid video capture technology accuracy. Orange box denotes testing zone for tracking software.
10.2.10 **Video Capture from Barnes Maze Apparatus showing Tracking Software View.**

![Video Capture from Barnes Maze Apparatus showing Tracking Software View](image)

Figure 10-18: Camera view of the Barnes Maze as tracked by ANY-maze™ (Stoelting Co., Wood Dale, IL, USA) software. A mouse is about to enter the exit hole. The area of the mouse is highlighted in blue, with an orange dot marking its centre point. The zone it is entering is highlighted in green. The zones it is occupying are listed in the top right hand corner. Orange circles delineate each of the potential exit holes, with a second, wider circle around the correct exit hole delineating the exit region.
10.3 **APPENDIX 3: DATA RELATING TO CHAPTER 5: BROMIDE REDUCES TREMOR AND DECREASES PERCEPTION OF STRESS INDUCED BY LOLITREM B INDUCED BK CHANNEL BLOCKADE**

10.3.1 **Comparison of Voluntary Movement in Lolitrem B (2mg/kg IP) Intoxicated Animals Treated with Different Therapeutics.**

![Bar graph comparing voluntary movement in lolitrem B intoxicated animals treated with different therapeutics. The x-axis represents different treatments: Bromide only, No treatment, Lolitrem B, Bromide + Lol B, Propanalol + Lol B, Alcohol + Lol B, Mefloquine + Lol B, and Carbenoxolone + Lol B. The y-axis represents distance (m). The graph shows significantly less movement in the Bromide only and No treatment control groups compared to the Bromide + Lol B treatment group (P≤0.0359). Only Bromide treatment improved movement compared to the positive control (Lolitrem B) group (P=0.0395).**

Figure 10-19: Comparison of voluntary movement on parallel rod in lolitrem B (2mg/kg IP) intoxicated animals, 1 hour post treatment with a potential therapeutic agent (n=4-11). For long acting drugs (mefloquine and bromide) measurement was one hour post lolitrem B injection. All treatments have significantly less movement than bromide only and no treatment controls (P≤0.0359). Only bromide treatment improved movement compared to positive control (lolitrem B) group (P=0.0395).
10.4 Appendix 4: Data Relating to Chapter 6: Development of a Model for Investigation of Perennial Ryegrass Toxicosis in Sheep

10.4.1 Basic Feed Analysis on Ryegrass Seed.

Table 10-2: Basic Feed Analysis on Ryegrass Seed

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<th>Wagga Wagga Feed Quality Testing Laboratory</th>
<th>Specimen Type: By Product</th>
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<th>Results</th>
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<th>LOR</th>
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<td>Neutral Detergent Fibre</td>
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<td>Acid Detergent Fibre</td>
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Comment(s): DMD = Dry Matter Digestibility
DOMD = Digestible Organic Matter in the Dry Matter

LOR = Limit of Reporting, the minimum quantity that can be reported with confidence.
10.4.2 Sheep showing Clinical Signs Consistent with Clinical Entry Criteria to Study.

Figure 10-20: Sheep showing signs clinical consistent with PRGT and meeting criteria for inclusion into Testing Phase of trial, namely dropping to sternal recumbency and showing signs of rhythmic myoclonus (rhythmic hyperextension of forelimbs) and poor limb sequencing (both forelimbs in extension, collapse in hindlimbs from failure to coordinate normal flexion and extension of limbs during rapid movement).
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## Testing Phase Day 2 (exit) Serum Biochemistry.

**Table 10-4: Testing Phase Day 2 Serum Biochemistry**

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Chapter 10.4 Appendix 4: Data Relating to Chapter 6: Development of a Model for Investigation of Perennial Ryegrass Toxicosis in Sheep
### Table 10-5: Feeding phase day 1 and testing phase day 2 urinalysis and PCV

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<th>PCV</th>
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10.5 Appendix 5: Patent for Treatment of Toxicosis in Sheep.

(12) United States Patent
(10) Patent No.: US 9,913,858 B2
(45) Date of Patent: Mar. 13, 2018

Quinn et al.

(54) PREVENTION AND TREATMENT OF TOXICOSIS

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(72) Inventors: Jane Quinn, New South Wales (AU); Scott Edwards, New South Wales (AU); Martin Combs, New South Wales (AU)

(73) Assignees: MEAT & LIVESTOCK AUSTRALIA LIMITED, North Sydney, New South Wales (AU); CHARLES STURT UNIVERSITY, Wagga Wagga, New South Wales (AU)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: 15/038,312

(22) PCT Filed: Nov. 20, 2014

(86) PCT No.: PCT/AU2014/050363

§ 371 (c)(1), (2) Date: May 20, 2016

(87) PCT Pub. No.: WO2015/074115

PCT Pub. Date: May 28, 2015

(65) Prior Publication Data

(30) Foreign Application Priority Data
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(51) Int. Cl.
A61K 33/00 (2006.01)
A61K 33/06 (2006.01)
A23K 20/20 (2016.01)
A23K 20/22 (2016.01)
A23K 50/10 (2016.01)
A61K 9/00 (2006.01)

(52) U.S. Cl.
CPC .............. A61K 33/00 (2013.01); A23K 20/20 (2016.05); A23K 20/22 (2016.05); A23K 50/10 (2016.05); A61K 9/0019 (2013.01); A61K 9/0053 (2013.01); A61K 33/06 (2013.01)

(58) Field of Classification Search
CPC .......... A23K 50/10; A23K 20/20; A23K 20/22; A23K 20/24; A61K 33/00; A61K 9/0019; A61K 9/0053

See application file for complete search history.

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U.S. Appl. No 15/038,261.

(74) Attorney, Agent, or Firm — Fisch, Even, Tabin & Flannery, L.I.P.

(57) ABSTRACT
The invention described in this specification relates to the prevention and treatment of alkaloid-induced toxicosis in pasture grazing animals.

11 Claims, 13 Drawing Sheets
Figure 1

A

B

C

D

U.S. Patent
Mar. 13, 2018
Sheet 1 of 13
US 9,913,858 B2
Figure 2

Motion to Tremor Ratio 1 hour post iolitren B injection 2mg/kg IP

- iolitren B
- iolitren B + Bravetac
- Bravetac Control
- Vehicle
Chapter 10.5, Appendix 5: Patent for treatment of toxicosis in sheep
Figure 4

Novel Arena: Distance moved at 210min post litiirem B injection 2mg/kg IP

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<td>Bransod + Litiirem B</td>
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Figure 5

![Diagram showing novel arena time freezing at 21hmin post belladonna B injection 2mg/kg IP.]
Figure 6
Figure 11
Figure 12

Pre and post treatment tremor intensity ratio

![Graph showing pre and post treatment tremor intensity ratio](image)
Chapter 10.5, Appendix 5: Patent for treatment of toxicosis in sheep

Figure 13

![Graph showing comparison of serum and CSF samples from different groups.](image-url)
Chapter 10.5, Appendix 5: Patent for treatment of toxicosis in sheep

1 PREVENTION AND TREATMENT OF TOXICOSIS

CROSS-REFERENCE TO RELATED APPLICATION

This application is a U.S. national phase application of International Application No. PCT/US2014/050365, filed Nov. 20, 2014, designating the United States, which claims priority to Australian Patent Application No. 2013/044317, filed Nov. 20, 2013, which are incorporated by reference herein in their entirety.

FIELD OF THE INVENTION

This invention relates to the prevention and treatment of alkaloid-induced toxicosis (such as lobellin B-induced toxicosis) in pasture growing animals.

BACKGROUND OF THE INVENTION

Rye grasses (genus Lolium) are commonly used to feed animals throughout the world. Such grasses can often be infected with endophyte fungi, such as those from the Neotyphodium genus. These fungi can live on these grasses and produce compounds (such as alkaloids) which can be useful to the plant. Some of these compounds can also be harmful to animals that consume the infected plant, and result in toxicosis.

One such example is the toxicosis resulting from the fungus Neotyphodium lolii, which produces the alkaloid lobellin (I) in perennial ryegrass (Lolium perenne). This toxin forms a major component of the toxicosis called perennial ryegrass toxicosis (PRGT), or "Ryegrass Staggers," and can lead to neuromuscular dysfunction with symptoms such as tremors, staggering, ataxia, hyperesthesia, tetany, gut abnormalities, increased severity of ill-coordination on movement and inful or continued sternal recumbency. These effects can be further exacerbated by external stimuli, and severe forms can lead to death of the animal, typically as a result of dehydration, starvation or attack by predators. Mild or effects of lobellin B-induced toxicosis can also have severe commercial outcomes, such as reduced live-weight gain, scarring and increased DM3 formation, reduced fertility and low milk yields. The toxicosis that affect ruminants grazing Panicum also causes tremors and other neurological and systemic clinical signs to those observed in respect of PRGT.

There is currently no known cure for treating animals with lobellin B intoxication, PRGT or Phalaris toxicosis and the subsequent animal morbidity and mortality impacts adversely on animal welfare and results in enormous losses in animal production revenue. Animals may recover from such conditions, but they can be moved away from the affected pastures. However, this is not always possible, especially where large pasture areas are infected and many animals are grazing on that pasture.

Whilst endophyte-infected pastures can be removed and replaced with grass that is not infected, this is of great effort and cost to achieve and a not always successful. The replacement grass may also not be as viable as the endophyte-infected pasture; hence it may be more prone, for example, to drought, due to the loss of the protective effects that the endophyte provides to the plants. Similarly, it is difficult to ensure that the endophyte-infected seed is completely removed from the pasture, which may result in re-infection. Removal and replacement of endophyte-infected pastures is therefore not always performed.

It is also difficult and time-consuming to test for the endophytes. Testing can only be performed by laboratory analysis, and representative samples taken from a large spread of the pasture need to be carefully collected and tested within a short timeframe. Testing is therefore not always practical and may not be accurate depending on factors such as the samples selected and submitted, and the transportation conditions of the samples to the laboratory.

There have been several other approaches investigated to overcome the problems of endophyte-toxicosis toxicosis. A grass modified to be unpalatable to endophyte infection may overcome the problem of pasture re-infection. However, ever, similarly to the replacement pastures, as the endophytes also provide the plant with benefits such as resistance to drought and protection from insects, this approach can still greatly disadvantage the plant and viability of the pasture.

Another approach has been to investigate the selective breeding or genetic modification of the endophyte to maximise its positive effects on the plant (e.g. insecticide properties) whilst minimising the expression of the toxicosis-causing alkaloids. These approaches, however, are expensive and have been shown to be not completely effective and/or commercially practical across all levels of farming.

Another approach includes feeding animals additives to overcome the toxicity of the alkaloids. An example of this is outlined in patent application WO 01/05728, whereby a modified yeast cell wall and a mineral clay is fed to animals to inactivate the endophyte alkaloids causing toxicosis by binding the alkaloids within the gastrointestinal tract (before their systemic absorption). This treatment does not interfere with the pharmaceutical of the endophyte and prevents its absorption. As such, this approach does not treat the symptoms of toxicosis, but can minimise the effect of the alkaloids on the animal.

While these approaches have each displayed some success, none have been able to significantly reduce the impact of alkaloid-induced toxicosis using a practical, commercially viable technique.

Reference to any prior art in the specification is not an acknowledgment or suggestion that this prior art forms part of the common general knowledge in any jurisdiction or that this prior art could reasonably be expected to be understood, regarded as relevant, and/or combined with other pieces of prior art by a skilled person.

SUMMARY OF THE INVENTION

This invention relates to the prevention and/or treatment of alkaloid-induced toxicosis in animals. In particular, it relates to the prevention and/or treatment of perennial ryegrass toxicosis, phalaris toxicosis and fascia grass toxicosis in animals that graze on these grasses.

In one embodiment, the invention provides a method of preventing an animal from grazing in a selected pasture. The method seeks to prevent symptoms of lobellin B toxicity from developing in the animal during, or at completion of, grazing in the pasture. The method includes the step of administering to an animal a formulation including bromide to an animal selected for grazing in a selected pasture to an animal effective for preventing the animal from developing symptoms of lobellin B toxicity. The formulation is to be administered to the animal before release of the animal to the pasture for grazing.
The present invention also provides a method of treating symptoms of lofotenin B toxicity in an animal, the method including administering bromide to an animal having symptoms of lofotenin B toxicity. In one embodiment, the symptoms have been acquired through pasture grazing.

The present invention also provides use of bromide in the manufacture of a formulation for preventing symptoms of lofotenin B toxicity from developing in an animal during, or at completion of, pasture grazing. The formulation is to be administered to the animal prior to release of the animal for pasture grazing.

The present invention also provides a method of treating symptoms of lofotenin B toxicity in an animal, the method including administering bromide to an animal having symptoms of lofotenin B toxicity. In one embodiment, the symptoms have been acquired through pasture grazing.

The present invention also provides use of bromide in the manufacture of a formulation for preventing symptoms of lofotenin B toxicity from developing in an animal during, or at the completion of, pasture grazing. The formulation for administration to the animal prior to release of the animal for pasture grazing.

In one embodiment, the bromide may be administered orally, intravenously or intraperitoneally. In another embodiment, the bromide is in the form of a salt such as potassium or magnesium bromide. Most preferably, the bromide salt is in the form of potassium or magnesium bromide, which are useful for oral administration. Sodium bromide is useful for intravenous administration.

The present invention also relates to a formulation for use in preparing an animal for pasture grazing to prevent symptoms of lofotenin B toxicity from developing in the animal during, or at completion of, grazing. The formulation including bromide in an amount for preventing symptoms of lofotenin B toxicity developing in the animal, the formulation for administration prior to release of the animal for pasture grazing.

The present invention also relates to a formulation for use in preparing an animal for pasture grazing to prevent symptoms of lofotenin B toxicity developing in the animal during, or at completion of, grazing. The formulation including bromide in an amount for preventing symptoms of lofotenin B toxicity developing in the animal, the formulation for administration prior to release of the animal for pasture grazing.

In one embodiment, the animal is a ruminant (e.g., ox, bovine or caprine).

Further aspects of the present invention and further embodiments of the aspects described in the preceding paragraphs will become apparent from the following description, given by way of example and with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Tremor analysis of animals treated with a single dose of lofotenin B toxin. Tremor analysis: FFT frequency analysis from a mouse prior to (A) and 6 hours post lofotenin B injection 2 mg/kg i.p. (B). Tremor frequency (0.07 Hz) power output represents primarily movement. (C) Tremor-Motion power ratio reveals tremor accounting for an increasing proportion of movement even at 6 hours. (D) Tremor-Direction power ratio at one hour post lofotenin B injection (n=4) and vehicle only control (n=4) (p<0.05, lofotenin B 2 mg/kg i.p. vs vehicle).

FIG. 2. Ratio of power output from tremor (0-20 Hz range) as compared to total power output (movement/tremor ratio) in lofotenin B treated animals.

FIG. 3. Peak tremor frequency one hour post lofotenin B injection.

FIG. 4. Distance traveled in animals treated with lofotenin B toxin with or without potassium bromide pre-treatment. Novel area test: Distance moved 3.5 hours post lofotenin B injection 2 mg/kg IP.

FIG. 5. Freezing episodes in animals treated with lofotenin B toxin with or without potassium bromide pre-treatment. Novel area test: Freezing time 3.5 hours post lofotenin B injection 2 mg/kg IP.

FIG. 6. Serum concentrations (mean±SD) of bromide after intravenous administration to eight sheep at a dose of 120 mg/kg.

FIG. 7. Serum concentrations (mean±SD) of bromide after oral administration to eight sheep at a dose of 120 mg/kg. Note multiple peaks occurring after 24 hours.

FIG. 8. Histological lesions observed in the cerebellum of lofotenin B intoxicated animals. Those clear histopathological lesions are nodule spheroids (*) and pyknotic nuclei (red arrowsheads) present in the granular layer and vacuolation within Purkinje neurons (white arrowshead).

FIG. 9. Serum concentrations in lofotenin B intoxicated sheep treated with an acute oral dose of potassium bromide.

FIG. 10. Time to falling in seconds of animals exposed to lofotenin B toxin only or toxin plus treatment with acute oral potassium bromide. Values shown for Group 1 (lofotenin B toxin only, n=8) and Group 2 (lofotenin 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.

FIG. 11. Mean composite gait score over time for animals exposed to lofotenin 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.

FIG. 12. Median Day 2 tremor intensity ratios for Groups 2 (lofotenin B intoxicated, no treatment) and 3 (lofotenin B intoxicated, acute KBr treatment). ** Significant difference between treated and untreated animals, p<0.01.

FIG. 13. Bromide levels in serum and CSF are higher in lofotenin B intoxicated animals than in un intoxicated controls. Serina: 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.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Reference will now be made in detail to certain embodiments of the invention. While the invention will be described in conjunction with the embodiments, it will be understood that the invention is not limited to the embodiments to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equival-
Chapter 10.5, Appendix 5: Patent for treatment of toxicosis in sheep

US 9,913,858 B2

leasts, which may be included within the scope of the present invention as defined by the claims.

One skilled in the art will recognize many uses and methods of treatment similar or equivalent to those described herein, which could be used in the practice of the present invention. The present invention is in no way limited to the specific methods and materials described.

It will be appreciated that the invention has two major applications. In one aspect, the invention will be used in the treatment of animals suffering from liliom B toxicity. In another aspect, the invention will be used in a prophylactic manner to prevent the development of the toxicosis symptoms in the animal. Prevention of such disorders may be particularly useful during peak seasons of endophilic growth, which can be predictable in some regions.

The invention is well-suited to preventing and treating toxicosis resulting from endophyte-infected or endophyte-prone grasses, such as perennial ryegrasses, *Phalaris* or fescue grasses. This includes toxicosis induced by compounds produced by endophytes (such as those from the *Neoshyllodium* genus), which can form a symbiotic relationship with such grasses. For example, the toxicosis may be due to *Neoshyllodium* infection of the grass.

The compounds inducing the toxicosis may be alkaloids. An example is an endole-herpovib, such as liliom B. Another example is an endole-alkaloid, such as endoleine. In particular, the invention relates to the treatment and prevention of symptoms caused by liliom B toxicity.

The invention can be used on any pasture-grazing animal that has developed, or is at risk of developing, symptoms of liliom B toxicity. In particular, the invention relates to the prevention and treatment of symptoms of liliom B toxicity in animals feeding on grass, and more particularly, perennial ryegrasses, *Phalaris* or fescue grass. Whilst this invention relates to any pasture-grazing animal, the invention is preferably directed towards ruminants including cattle, sheep, goats, camels, horses, alpacas and deer. In a certain embodiment, the animal may be a herbivore such as a horse or a rabbit. The invention is also not intended to cover insects, therefore, in one embodiment, the animal is not a human.

As mentioned above, symptoms of liliom B toxicity in cattle, sheep, goats, and rabbits, occur as a result of the presence of alkaloids in the animal’s diet. These alkaloids accumulate in the animal, and may cause a variety of health-related abnormalities, including increased deaths, liver dysfunction, and reduced fertility. An animal having these symptoms, as well as others that are recognized as being indicative of POGT, can be treated according to the present invention. Therefore, in one embodiment, the animal is first assessed as having developed, or being likely to develop, symptoms of liliom B toxicity. Diagnosis or likely development of POGT and liliom B toxicosis may be determined by any one of the following: specific toxicological testing of pasture samples, the Kugel test, and/or the presence of severe neurological signs consistent with POGT, and/or by histopathology findings consistent with a diagnosis of POGT. This may be further indicated by the absence of clinical or neurological signs indicating any other disease intoxicology. A person skilled in the art will understand that “symptoms”, as used herein, is the equivalent of “clinical signs.”

Successful treatment of the animal will be achieved when one or more of the symptoms mentioned above are completely or partially resolved. For example, the animal’s symptoms may cease completely, while any stingers exhibited by the animal may decrease to the point where they are no longer a concern for the animal’s ability to function normally (e.g. the animal is able to access drinking water and feed itself). In addition, the growth performance of the animal will improve (e.g. the animal’s weight, reproductive performance and milk production will return to normal levels, and tumor and movement abnormalities will resolve).

In the context of prevention, the animal to be administered bromide will be any pasture-grazing animal that is at risk of developing symptoms of liliom B toxicity. That is, the animal is one that may through grazing on grass infected with endophyte flanks, develop symptoms of toxicosis. Therefore, in one embodiment, the animal is first assessed as being at risk of developing symptoms of liliom B toxicity. To prevent toxicosis, a pasture-grazing animal will therefore generally be given bromide before it is released into a pasture for grazing.

In one embodiment, the animal does not have stress-related or stress-induced inappetence at the time of administration of bromide. In one embodiment, the feed intake of the animal up to the time of administration of bromide has been normal. In one embodiment, the animal is not suffering from grass toxicosis at the time of administration of bromide. In one embodiment, the animal does not have a movement disorder at the time of administration of bromide. As mentioned above, peak seasons of endophyte growth are predictable in some regions, and therefore bromide may only be administered to an animal during particular times of the year. In one embodiment, bromide (e.g. a formulation containing bromide) is administered for a period of one day to no more than two weeks from release of the animal to the selected pasture for grazing on a daily basis, for example, for seven to 10 days before the animal is released into a pasture (i.e. before grazing). It may also be administered after release (for example, for several months) of the animal into the pasture (i.e. during grazing). For example, the bromide may be administered to the animal for one month, two months, three months, four months, five months, or more, after release of the animal into the pasture. Further, bromide may be administered for up to 14 days after the animal has been removed from the pasture and/or on one or more occasions.

Bromide may also be administered via a slow-release intra-ruminal capsule. Successful prevention of toxicosis will be achieved when the animal does not develop any, or only develops to an insignificant extent, symptoms of toxicosis. In addition, the growth performance of the animal will not be adversely affected (e.g. the animal’s weight and milk production will remain at normal levels, the reproductive performance will not be impaired, and conditions such as fescue foot will not develop). A dose of a bromide-containing formulation of the invention may be delivered at once, for example, as a bolus, or over the course of several hours. Theoretically, there is no known limit to the dosage provided that the bromide does not cause an animal to lose weight, thereby diminishing the quality of the animal or products therefrom.

The bromide may be provided in the form of a formula containing bromide. As described herein, the specificity of the bromide level of bromide for any particular animal may depend upon a variety of factors including the activity of the specific
Chapter 10.5, Appendix 5: Patent for treatment of toxicosis in sheep

US 9,913,858 B2

7 bromide, employed, the age, body weight, general health, sex, and/or diet of the animal, time of administration, route of administration, and rate of excretion, drug or supplement combination (i.e. other drugs or supplements being used concurrently with the bromide), and the severity of the toxicosis being exhibited, if being used as a treatment.

To prevent or treat symptoms of 5 iotin B toxicity, bromide (e.g. in a formulation including bromide) may be administered to the animal in an amount of about 10 to about 750 mg/kg per dose. For example, the bromide may be administered to the animal in an amount of about 10 to about 650 mg/kg, or about 100 to about 500 mg/kg. A dose of about 10 to about 500 mg/kg is preferred for intravenous administration. A dose of about 10 to about 750 mg/kg is preferred for oral administration. Bromide (e.g. in a formulation including bromide) may be administered to the animal in an amount of about 10 mg/kg, about 20 mg/kg, about 50 mg/kg, about 75 mg/kg, about 100 mg/kg, about 120 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300 mg/kg, about 500 mg/kg, about 600 mg/kg, about 750 mg/kg, or about 900 mg/kg per dose.

In one embodiment to prevent or treat symptoms of 20 iotin B toxicity, the bromide (e.g. in a formulation including bromide) is administered in an amount to provide an animal with about 10 to about 750 mg/kg animal weight of bromide. For example, the bromide may be administered or provided in an amount to provide the animal with from about 20 to about 600 mg/kg animal weight (e.g. from about 40 to about 500 mg/kg animal weight, from about 60 to about 400 mg/kg animal weight, or from about 100 to about 300 mg/kg animal weight) of bromide. In one embodiment, the bromide is in an amount to provide an animal with about 500 mg/kg animal weight of bromide.

Bromide (e.g. in a formulation including bromide) may be administered to provide an animal with about 10 mg/kg, about 20 mg/kg, about 50 mg/kg, about 75 mg/kg, about 100 mg/kg, about 120 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300 mg/kg, about 400 mg/kg, about 500 mg/kg, about 550 mg/kg, about 600 mg/kg, about 750 mg/kg, or about 900 mg/kg per dose for intravenous administration.

In one embodiment, the formulation comprises bromide in an amount to provide an animal with about 10 to about 500 mg bromide/kg animal weight of bromide. In one embodiment, the formulation includes bromide in an amount to provide an animal with about 20 to about 400 mg/kg animal weight (e.g. from about 30 to about 300 mg/kg animal weight) of bromide. In one embodiment, the formulation includes bromide in an amount to provide an animal with about 500 mg/kg animal weight of bromide.

Preferred drenches are those adapted for use in a ruminant animal, particularly sheep (i.e. ovine) or cattle (i.e. bovine). Preferably, the concentration of bromide in the drench is from about 5 to about 70% w/v (liquid or paste formulation) and about 5 to about 70% w/w (powder or solid formulation). In one embodiment, the concentration of bromide in the drench is from about 10 to about 60%, from about 20 to about 50%, or from about 30 to about 40% w/v or w/w. In one embodiment, the concentration of bromide in the drench is in about 5, about 10, about 20, about 30, about 40, about 50, about 60 or about 70% w/v or w/w.

In one embodiment, the active ingredient in the drench is bromide.

In another embodiment the formulation provides an injectable formulation including bromide, wherein the formulation comprises bromide in an amount to provide an animal with about 10 to about 500 mg bromide/kg animal weight of bromide. In one embodiment, the injectable formulation includes bromide in an amount to provide an animal with about 20 to about 400 mg/kg animal weight (e.g. from about 30 to about 300 mg/kg animal weight, from about 50 to about 400 mg/kg animal weight or from about 100 to about 300 mg/kg animal weight) of bromide. In one embodiment, the injectable formulation includes bromide in an amount to provide an animal with about 500 mg/kg animal weight of bromide.

In one embodiment, the injectable formulation includes bromide in an amount to provide an animal with about 10 mg/kg, about 20 mg/kg, about 50 mg/kg, about 75 mg/kg, about 100 mg/kg, about 120 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300 mg/kg, about 400 mg/kg, about 500 mg/kg, about 550 mg/kg, about 600 mg/kg, about 750 mg/kg, or about 900 mg/kg per dose for intravenous administration.

In one embodiment, the injectable formulation includes bromide in an amount to provide an animal with about 10 to about 50 mg/kg (for oral administration) and about 0.4 to about 25 mg/kg (for intravenous administration).

In one embodiment, bromide is the only active ingredient in the formulation. In one embodiment, bromide is the only active ingredient provided to the animal. Therefore, in one embodiment, the formulation does not include providing therapeutic or nutritive agents, other than bromide, to the animal. Examples of such agents include electrolytes (e.g. sodium, potassium, magnesium, manganese, chromium, and calcium, and chloride, carbonate, and water soluble sulfate salts thereof), amino acids (or salts thereof), and sources of energy (e.g. sugar). In one embodiment, the injection does not include providing a modified yeast cell wall and a mineral ingredient to the animal.

Suitable formulations for use in the present invention include drenches, gels, pastes, tablet/coated tablets, solution, gelatin capsules, injectable formulations, or intramuscular devices for slow release of the active.

In one embodiment the formulation provides a drench including bromide, wherein the drench comprises bromide in an amount to provide an animal with about 20 to about 600 mg bromide/kg animal weight. In one embodiment, the drench includes bromide in an amount to provide an animal with about 20 to about 600 mg/kg animal weight (e.g. from about 30 to about 300 mg/kg, from about 50 to about 400 mg/kg, or from about 100 to about 300 mg/kg animal weight) of bromide. In one embodiment, the drench includes bromide in an amount to provide an animal with about 300 mg/kg animal weight of bromide.

In one embodiment, the drench includes bromide in an amount to provide an animal with about 20 mg/kg, about 50 mg/kg, about 100 mg/kg, about 120 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300 mg/kg, about 400 mg/kg, about 450 mg/kg or about 500 mg/kg animal weight of bromide.

Preferably, the formulation is adapted for application to sheep or cattle. Preferably the concentration of bromide is from about 5 to about 70% w/v. In one embodiment, the concentration of bromide in the formulation is from about 10 to about 60%, from about 20 to about 50%, or from about 30 to about 40% w/v. In one embodiment, the concentration of bromide in the formulation is from about 5, about 10, about 20, about 30, about 40, about 50 or about 70% w/v.

The drench or injectable formulation may be provided in the form of a kit including written instructions enabling use of the kit in a method described above. In one embodiment, the kit includes a drench, as described herein, and written instructions enabling use of the kit in a method described herein.

In one embodiment, the kit includes a drench, as described herein, and written instructions enabling use of the kit in a method described herein.

236
Chapter 10.5, Appendix 5: Patent for treatment of toxicosis in sheep

US 9,913,858 B2

The bromide may be provided in the form of a supplement in liquid (e.g., an aqueous solution) or solid form that is to be consumed by the animal. It will be evident that, given the variable consumption of solids and liquids by animals, the amount of bromide in a consumable composition as stated above is approximate and may be varied depending on the type of formulation (solid vs. liquid), the solubility of the bromide, the body weight of the animal and the average solid and liquid intake of the animal. Supplements may be provided with carriers, or may be formulated into feed with binders. Exemplary carriers include grains or grass by-products, such as oats, barley, wheat, canary, rye, sorghum, millet, corn, legumes and grasses.

In one embodiment, bromide is provided in a fixed amount of between about 0.1 to 5% w/w dry matter. For example, bromide may be provided in an amount of between about 0.1 and 4% or 1% and 3% w/w dry matter. In one embodiment, the concentration of bromide in feed is about 0.01, about 0.2, about 0.5, about 1, about 3, about 4 or about 5% w/w dry matter.

The bromide may be administered with another medication (e.g., an antibiotic), a growth promoter or incorporated into a mineral pre-mix. Suitable amounts of bromide in this regard include between about 0.1 and about 60% w/w dry weight. For example, bromide may be provided in an amount of between about 0.1 and about 50%, about 1 and about 40%, about 5 and about 30% or about 10 and about 20% w/w dry weight.

In one embodiment, the concentration of bromide is about 0.1, about 0.2, about 0.2, about 1, about 2, about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 45, about 50 or about 55 or about 60% w/w dry matter. Specific examples include:

1. IV injection—single bolus high dose of Naline for acute use; may be used to have a bromide in administration of 10 to 70% w/w. Bromide dose: 10 to 500 mg/kg.

2. Oral—for a renmal donor (single phase release liquid, paste or solid formulation) fast release complete within 6 to 24 hours. For prophylaxis or treatment, dose lasting up to 3 weeks. This can be given at the observation of mild clinical signs or as a prophylactic dose prior to PRGT critical periods. Dose to contain bromide 10-70% w/w. Bromide dose: 10 to 500 mg/kg.

3. Oral—intramuscular device—dose or dual release formulation for priming dose and slow release for maintenance of dose lasting up to 6 weeks. This can be given at the observation of mild clinical signs or as a prophylactic dose prior to PRGT critical periods. Formulation to contain bromide 10 to 70% w/w or w/w. Bromide dose: 100 mg/kg.

4. Oral—in a feed additive—the bromide may be provided in the form of a supplement in liquid or solid form that is to be consumed by the animal. It will be evident that, given the variable consumption of solids and liquids by animals; the amount of bromide in a consumable composition is approximate and may be varied depending on the type of formulation (solid vs. liquid), the solubility of the bromide, the body weight of the animal and the average solid and liquid intake of the animal. Supplements may be provided with carriers, or may be formulated into feed with binders. Exemplary carriers include grains or grass by-products, such as oats, barley, wheat, canary, rye, sorghum, millet, corn, legumes and grasses. Inclusion of bromide in an antibiotic or mineral pre-mix at 0.1 to 60% w/w is a practical method of administering bromide to achieve a dose of 10 to 750 mg/kg. Overall, bromide is incorporated into the ration at a rate of 0.01 to 5% w/w dry matter. This delivery can be used after, or in addition to, deliveries 1 & 2.

5. In an on-farm outbreak of PRGT, animals might receive on a basis of individual need a combination of bromide 10 to 500 mg/kg dose, depending on progression of their disorder and/or anamnestic of their clinical signs on return to pasture. As used herein, “bromide” refers to the bromine ion (Br-).

It will be understood by a person skilled in the art that bromide will generally be administered to the animal in the form of a salt and/or other compound with a bromine group that readily dissociates in situ and/or in vivo to give bromide. Preferably, the bromide is in the form of a salt, which may contain an alkali metal or alkaline earth metal. For example, the bromide may be in the form of potassium bromide, sodium bromide or magnesium bromide. Potassium bromide and magnesium bromide are particularly useful for oral administration and sodium bromide is useful for intravenous administration. Magnesium bromide has a higher percentage of bromide than both the potassium and sodium salts, and possesses much greater water solubility than both the potassium and sodium salts. Consequently, magnesium bromide solutions can be made at much higher bromide concentrations than other salts, before saturation is reached. This property of the magnesium salt of bromide impacts on formulation; low volume delivery is practically and economically advantageous.

Bromide may be administered to an animal by several different routes, including those selected from orally, nasally, osseously, vaginally, rectally, topically, subcutaneously, intramuscularly, intravenously, intraperitoneally and intradermally. Preferably, the bromide is administered to the animal orally.

In one embodiment, the formulation includes, in addition to the bromide, one or more pharmaceutically acceptable excipients, such as binders, disintegrants, dispersants, lubricants, colours, flavors, coatings, gelling agents, absorption-enhancing agents, emulsifiers, surfactants, buffers, building agents, toxicity-adjusting agents, preservatives, sweetening agents, solvents, and sweeteners. Suitable examples of excipients to include in formulations for use in the invention are well known to a person skilled in the art. In one embodiment, the bromide is administered or given to the animal in an aqueous solution. For example, the bromide can be dissolved in the animal's drinking water for the animal to consume. The bromide may be incorporated into animal feed as a means to administer the bromide to the animal and/or encourage the animal to consume the bromide (as discussed above). Animal feed includes any food that the animal is capable of ingesting and if desired, any additional ingestible materials. For example, it could include grass, plant extracts, vitamins, minerals, feed supplements and other such materials.

In one aspect, the invention provides a pharmaceutical composition including an effective amount of bromide and one or more pharmaceutically acceptable excipients for use in preventing symptoms of livestock injury from arising in an animal selected for pasture grazing. The present invention also provides a pharmaceutical composition including an effective amount of bromide and one or more pharmaceutically acceptable excipients for use in preparing an animal for pasture grazing to prevent symptoms of livestock injury from arising in the animal during, or at completion of, grazing.
Chapter 10.5, Appendix 5: Patent for treatment of toxicosis in sheep

US 9,913,858 B2

11 The invention also relates to the use of a therapeutically effective amount of bromide for preventing an animal for grazing in a selected pasture to prevent symptoms of lolidrium B toxicity from developing in the animal during or at completion of grazing, including administering a formulation containing bromide to the animal selected for grazing in a selected pasture in an amount effective for preventing the animal from developing symptoms of lolidrium B toxicity before release of the animal to the pasture for grazing.

The present invention also relates to a composition having an active ingredient for use in preparing an animal for grazing in a selected pasture to prevent symptoms of lolidrium B toxicity from developing in the animal during, or at completion of, grazing in the pasture, wherein the active ingredient is bromide.

The present invention also relates to a method of preparing an animal for grazing in a selected pasture to prevent symptoms of lolidrium B toxicity from developing in the animal during, or at completion of, grazing in the pasture, wherein the active ingredient is bromide.

In one embodiment, bromide is the only active in the formulation or composition.

The present invention also relates to a composition having an active ingredient for use in preparing an animal for grazing in a selected pasture to prevent symptoms of lolidrium B toxicity from developing in the animal during, or at completion of, grazing in the pasture, wherein the active ingredient is bromide.

The present invention also relates to a method of preparing an animal for grazing in a selected pasture to prevent symptoms of lolidrium B toxicity from developing in the animal during, or at completion of, grazing in the pasture, wherein the active ingredient is bromide.

In one embodiment, bromide is the only active in the formulation or composition.

The present invention also relates to a formulation containing bromide for preventing an animal from developing symptoms of lolidrium B toxicity before release of the animal to the pasture for grazing.

EXAMINES

Examples 1

1.1 Lolidrium B was tested in mice to establish its as a suitable model for potential rye grass toxicosis and lolidrium B introduction in grazing animals. Lolidrium B (2 mg/kg) administered intraperitoneally (IP) produced a decrease in movement and a distinctive tremor spike in the range of 16-19 Hz. These signs are consistent with clinical signs seen in grazing animals (See FIG. 1). Example 2

2. Bromide Alleviates/Prevents Clinical Signs of Lolidrium B Toxicosis in a Rodent Model of ROGRT.

To investigate the potential of bromide as a therapeutic agent for perennial rye grass toxicosis and lolidrium B intoxication in grazing animals, mice were administered 2500 ppm KBr in their drinking water for 7 days prior to injection with lolidrium B (2 mg/kg IP). Mice demonstrated reduced
tremor in movement into and reduced peak tremor frequency at one hour post injection. Animals tested in a novel arena test at 3.5 hours post lidocaine B injection (2 mg/kg IP) demonstrated increased movement and reduced time freezing. This provides strong evidence that bromide can alleviate or prevent clinical signs of presynaptic nigrostriatal toxicity and lidocaine B intoxication (see 10.0/5.2 to 5.3).

Example 3

Determination of Pharmacokinetics of Bromide in Sheep after Single Intravenous (IV) and Oral (PO) Doses

Sixteen Merino sheep were randomly assigned to two treatment groups. The intravenous (IV) group were given 120 mg/kg bromide, as sodium bromide. The per os (PO-oral) group were given 120 mg/kg bromide, as potassium bromide. Serum bromide concentrations were determined by colorimetric spectrophotometry.

Animals

Sixteen sheep, weighing between 40.5 kg and 67 kg and with an average body condition score of 2 to 2 were randomly divided into equal number (IV and PO treatment groups). Animals were placed in individual feeding pens and were fed twice daily on a ration of oats and lupins as well as ad libitum hay and water. Estimated chloride content for oats and lupins was 0.1% and 0.4% respectively.

Indwelling intravenous canulae (Braun, Certo Splitform canal 335, 16 gauge, 32 cm) were placed into the left jugular vein and secured with 2/0 polypropylene suture. A 25 cm low volume IV extension set (BDM TUTA, 25 mm minimal volume IV extension set) was connected to the catheter hub and the area was then bandaged.

Sodium bromide (NaBr) (Sigma-Aldrich) and potassium bromide (KBr) (Sigma-Aldrich) solutions were prepared using sterile water. The prepared NaBr solution was then filtered through a microfilter (0.22 μm MILLIEX, GE, Cork, Ireland). Sodium and potassium salts were administered to dose sheep with 120 mg/kg of Br (156.4 mg/kg NaBr or 170.8 mg/kg KBr). All serum concentrations are for Br. Potassium bromide is the most readily available form of Br for oral therapy. Sodium bromide was used for the IV study because of cardiotoxicity associated with potassium bromide. Both salts were identically dissociated in solution therefore no PK differences were expected.

IV Bromide

NaBr solution was administered through the cephalic vein using a 21 gauge needle over a period of 1 minute. Sheep were restrained in a seated position. Blood samples were collected at 0, 1, 5, 10, 15, 30 and 60 min and then at 1, 2, 3, 4, 6, 8, 10, 12, 24 h. Samples thereafter were collected at 12 h intervals to 240 h then at 24 h intervals to 356 h. A final sample was taken at 528 h. For each sample, the initial 2 ml of blood collected was discarded and a sterile syringe used to withdraw 5 ml of blood which was then placed into a plain separator blood tube (Vacutainer: Cenost Boro-one). The cannula was flushed with 5 ml of 5% heparinized saline after each collection. Each blood sample was left to stand for 30 min before centrifugation at 2000 g for 5 min. Serum was harvested and stored at −20°C until analyses.

PO Bromide

KBr solution was administered via an orogastric tube, then flushed with 500 ml water. Blood samples were collected at 0, 2, 3, 4, 6, 8, 10, 12, 24 h, then at 12 h intervals to 240 h then at 24 h intervals to 356 h. A final sample was taken at 504 h. When collecting blood samples

at 1 h through to 10 h the rumen was aspirated, over the cefazolin HCl byc, to determine if the high level led affected rumen motility.

Determination of Serum Bromide Concentrations

Bromide concentrations were determined by colorimetric spectrophotometry as previously described (Hirtz, 1976, with some modifications). Briefly, 0.5 ml of 3% serum was added to 3.15 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 0.9% AuCl3 (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, R2=0.9992. The lower limit of quantification was 25 μg/ml.

Pharmacokinetics

Maximum concentration (Cmax) of Br and time to Cmax (Tmax) were determined directly from the data. Other PK parameters were determined for each sheep by use of non-compartmental analysis with a commercial software program (Gpdl 2.0, Gacov Thomas Verlag). Area under the curve (AUC(0-∞)) and area under the first moment curve (AUMC(0-∞)) were calculated by the linear trapezoidal rule (Gibaldi, 1982) the terminal elimination rate constant (λz) was calculated by means of log-linear regression. Whereas parameters Cmax, Tmax and AUC (where bioavailability is not absolute) are expected to differ when given IV or PO, λz should be the same, regardless of route of administration. A test of the hypothesis of no difference between the λz

population means was performed. All results are expressed as mean+standard deviation (SD).

After IV administration the maximum concentration (Cmax) was 823.13 ± 110.39 μg/ml, area under the curve (AUC(0-∞)) was 93.95 ± 13.53 μg/ml, and the time of maximum concentration (Tmax) was 100.1 ± 125.4 h. The terminal half-life (T1/2) of bromide after IV or PO administration was 71.2 ± 15.35 h and 340.7 ± 64.45 h, respectively. The bioavailability (F) of bromide was 92%. No adverse reactions were noted during this study in either treatment group. The concentration versus time profiles exhibited secondary peaks, suggestive of gastrointestinal residue redistribution of the drug.

All sheep in the PO group exhibited no discernable neurological effects. There was no observed alteration in rumen motility and animals continued to eat and drink. Assessment of any acute neurological effects correlating with peak Br concentration following IV administration was difficult as the sheep were held in the seated position throughout the initial 20 min, for ease of sampling. All IV sheep walked back to their individual pens and subjectively observers reported a mild tranquilising effect for approximately 1 to 2 h post treatment.

The relevant non-compartmental pharmacokinetic parameters derived from this study are summarised in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean (SD)</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (μg/ml)</td>
<td>823.13 ± 110.39</td>
<td>93.95 ± 13.53</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>100.1 ± 125.4</td>
<td>71.2 ± 15.35</td>
</tr>
<tr>
<td>AUC(0-∞) (μg/ml)</td>
<td>93.95 ± 13.53</td>
<td>340.7 ± 64.45</td>
</tr>
<tr>
<td>λz (h)</td>
<td>0.18 ± 0.24</td>
<td>0.19 ± 0.24</td>
</tr>
<tr>
<td>AUMC(0-∞) (h)</td>
<td>157.22 ± 15.33</td>
<td>143.90 ± 18.51</td>
</tr>
</tbody>
</table>

TABLE 1


Chapter 10.5, Appendix 5: Patent for treatment of toxicosis in sheep

US 9,913,858 B2

<table>
<thead>
<tr>
<th>TABLE 1-continued</th>
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</thead>
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<thead>
<tr>
<th>Patent 9,913,858 B2</th>
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15

<table>
<thead>
<tr>
<th>Performance Parameter (mean ± SD) after intravenous administration</th>
<th>Oral administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>1.50 ± 0.123</td>
</tr>
<tr>
<td>V1 (L/h)</td>
<td>1.260 ± 0.031</td>
</tr>
<tr>
<td>V2 (L)</td>
<td>2.594 ± 0.127</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>6.30 ± 0.123</td>
</tr>
</tbody>
</table>

The concentration-time profiles for IV and PO serum bromide were shown in Figures 6 and 7, respectively.

The t1/2 of IB in sheep following PO administration was 14.4 h; the IV t1/2 was 16.2 h; however, the difference between groups was not statistically significant (T=0.7832, df=14, p=0.4466). Because of the long 1% following PO administration, the serum IB concentration fluctuated within a narrow range (approximately 200-400 mg/L) for 14 h. This long t1/2 with two fold difference between peak and trough values over a two week period indicates that bromide would be useful for prophylactic use.

An interesting finding in this study was the numerous pronounced peaks in the PO concentration versus time curve following the initial absorption phase. These peaks were observed in all the 

1. The mean bioavailability of IB in this trial was 92.5%, which is desirably high.
2. The V2 value of 0.28 ± 0.013 reflects the ECF space (although volume of distribution figures are not primary measures of physiological compartments, they do often correlate well). The calculated volume of distribution at pseudo-equilibrium (V2), when equilibration with plasma fluid is assumed, was 0.304 ± 0.026 and 0.306 ± 0.026 mg/kg for IV and PO administration, respectively. That these measures are similar is reassuring in V2 is a proportionality factor relating concentrations during the log-linear phase of drug elimination, from which t1/2 is derived.

Volume of distribution is the parameter used to calculate a loading dose (LD) using the equation: LD = X0/C0, where C0 is concentration steady state, or the effective concentration for a particular use (as determined by PD studies). The V2 value is most appropriate where bromide is to be given as a PO bolus, and V1 is circumstances where it is to be given over a few days.

When administered PO bromide in sheep has a long half-life (t1/2) of approximately 14 days, with good bioavailability.

Example 4

Summary

Potassium bromide as a treatment for PEPT in sheep was evaluated using two delivery methods: 1) a single dose for treatment of acute seizures 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 blood samples were taken for clinical pathology, urine for specific gravity (USG) and electromyography for determination of normal abdominal 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 0.16 mg/kg body weight (BW) lithium (Li) toxic rising to 0.27 mg/kg BW after 21 days exposure to toxic feed. Toxicosis was delivered as pentaerythritol tetranitrate (PENT) at an amount of 10 mmol/kg BW as a bolus of 0.25 mmol/kg BW daily. A treatment group fed both lithium and PENT to all animals for 22 days and a treatment group only given no toxic feed but delivered the prophylactic dose of potassium bromide.

Clinical signs were observed in all these toxic feed groups. The first observable signs of lithium bromide 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 inactivity to noise, touch or movement. Intention tremor became increasingly marked as toxicosis 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 (dystrochokokinesia), lumpy-tongued gait and failure to maintain an appropriate direction. This transpired over a period of days to Type 2 movement disorder (Crauls, Randell et al 2014) defined by increasing rigidity of limbs on forced movement resulting finally in toxic and hindlimb extension with the animal collapsing into sternal or lateral recumbency (Combs, Randell et al 2014). Type 2 animals were able to recover and regain standing within a short period of time but would continue to fall if encouraged to move again. Identification of Type 2 movement disorder and day of falling was determined to be the date for initiation of treatment for the acute treatment group. Time to falling was recorded for all animals and ranged from immediate upon encroachment to moving to approximately 90 seconds only as encouragement to move again. 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 300 mg/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 of 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).

240
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 deviations in creatinine and urea observed but not outside animal 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 brains of intoxicated animals, these included small numbers of spheroids located in the granule cell layer, mainly in the lateral cerebellum, as well as pyleastic granule cell nuclei and occasional vaculated Purkinje neurons. These changes represent the earliest lesions of PRGT reported.

Animals in the potassium bromide prophylactic group also showed improved movement 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 confirms potassium bromide as an effective therapy and prophylactic agent for neurologically PRGT, or ‘Dry Grass Sluggers’ agents for neurological PRGT.

Methodology

1.1 Animals

Animals were White Sindu-Merino first cross male lambs between the ages of 8-12 months (n=45, live weight 36.3±2.02 kg) sourced from a single producer. They had been maintained under normal husbandry conditions gaining 1500g/day live weight and 180mm of stubble pastures prior to entry to the trial. No animals used exhibited any obvious pre-existing pathology. 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.

1.2 Toxic Feed

Toxic feed consists of seed containing toxic levels of lolitrem B without confounding high levels of ergovaline, a novel endophyte in perennial rye grass seed (Lolium AR8, Grassland Technology Ltd, New Zealand) was sourced. Toxic analyses by LC/MS was carried out by Agilent, New Zealand, which showed the seed to contain 11.1 mg/kg DM lolitrem B toxin. No ergovaline was detected.

On entry to the trial, all animals receiving toxic feed were exposed to a diet consisting of experimental ryegrass seed with a final lolitrem B concentration of 0.088 mg/kg LW, lucerne chaff and noodles 15g/kg/day for 3 days. After this induction period, the toxic content of the feed was increased to 0.16 mg/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. Ryegrass seed was constituted up to 65% of available feed on offer (TOF) by dry weight contributing a metabolisable energy of 12.1 MJ/kg DM.

1.3 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 pupillary light and menace reflexes. Electromyography (EMG) of three head muscles was performed. Urinalysis (urine) 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 hand position changes in normal consistency, feeding behaviour or water consumption, observable tremor of the head or body, head position (stiffness) and movement (incoordination), locomotory 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.

1.4 Treatments

1.4.1 Treatment Rates for Potassium Bromide

The anticonvulsant range of bromide in monogastric species is 0.8 to 20 mg/ml (Podell and Ferrar 1993). 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)=4×target concentration, and factoring in the 92% oral bioavailability of bromide in sheep, a LD of 340 mg/kg was obtained as an acute treatment.

Prophylactic sheep received a split loading dose of 300 mg/kg on day 1, 120 mg/kg (100 mg I.D.20 mg/kg daily maintenance dose) on day 2, 120 mg/kg on day 3 and 20 mg/kg daily for the duration of the trial. An acute treatment dose of 300 mg/kg LW was used for the trial. Both prophylactic and acute treatment doses were envisaged to give serum concentrations of 750 to 1000 μg/ml for the duration of the initial weeks of the trial.

1.4.2 Establishment of Treatment Groups

There were five treatment groups:

1. Negative Control: lucerne chaff only;
2. Positive Control: lucerne chaff containing 0.16 mg/kg LW lolitrem B;
3. Acute potassium bromide (KB) treatment: lucerne chaff containing 0.16 mg/kg LW lolitrem B, treated orally with 300 mg/kg bromide (Sigma Aldrich) on day of falling;
4. Prophylactic KB treatment: lucerne chaff containing 0.16 mg/kg LW lolitrem B, treated orally with a prophylactic therapeutic dose of potassium bromide with a loading dose given on day 1, followed by a maintenance dose administered orally daily (see below);
5. Prophylactic KB treatment control: lucerne chaff only, treated orally with prophylactic potassium bromide as described above.

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

Animals entered the trials in cohorts of five, each cohort containing one animal from each treatment group with entry occurring over three consecutive days such that groups of 15 animals undertook trials together. Three sets of 15 animals were used for this study (n=45) with each treatment group containing nine animals. Animals were maintained on the treatments as described above for the duration of the trial period.
Chapter 10.5, Appendix 5: Patent for treatment of toxicosis in sheep

US 9,913,858 B2

19 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 Day 1: animals were subjected to a full clinical examination, gait analysis, urinal 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, urinal and blood collection, neurological examination and EMG as well as live weight recorded prior to necropsy.

1.5. Electromyography

Hair samples were taken on entry to the trial, entry to the end of trial (Day 1) and post mortem (Day 3) using PowerLab™ (ADInstruments, Castle Hill, Australia) and data recorded using LabChart™ software (ADInstruments, Australia). In preparation, the fleece was shaved from an area over the triceps muscle along the brachiocephalic muscle of the neck. EMG electrodes were attached to the skin using SuperSleev™. Muscle activity was recorded for a minimum of three minutes with the animal standing at rest with a head pass filter set at 100 Hz. Three minute EMG recordings were taken using Ag–AgCl surface electrodes over the triceps muscle on entry to the trial, on first day of falling (Day 1) and 48 hours 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 30 Hz to estimate tension intensity.

1.6. 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 (five 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 while 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 1 movement disorder is defined as an impaired, alternating movement dyskinesia (dyskinesia). Brandy-hopping gait and failure to maintain an appropriate direction. Type 2 movement disorder is defined by increasing rigidity of limbs on forced movement resulting finally in tonic face and hind leg extension with the animal collapsing into sternal or lateral recumbency (Combs, Reindel 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 2 and a composite score noted for each animal for each day during the end of trial protocol. A total score of 30 was determined for each animal. The higher the score the greater the locomotory disturbance. Time to falling was determined post trial by analysis of video material.

### Table 2

<table>
<thead>
<tr>
<th>Clinical observation</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyskinesia</td>
<td>1</td>
</tr>
<tr>
<td>Mild distorted front</td>
<td>2</td>
</tr>
<tr>
<td>hind limb coordination</td>
<td>3</td>
</tr>
<tr>
<td>Incoordination</td>
<td></td>
</tr>
<tr>
<td>Ataxia</td>
<td>1</td>
</tr>
<tr>
<td>Mild wide-based</td>
<td></td>
</tr>
<tr>
<td>gait only</td>
<td></td>
</tr>
<tr>
<td>Stumbling without</td>
<td>2</td>
</tr>
<tr>
<td>falling on motor</td>
<td></td>
</tr>
<tr>
<td>movement</td>
<td></td>
</tr>
<tr>
<td>Clinical mixture</td>
<td>Score</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

20 Changes in foot and hind limb coordination mark as pacing at strobic Jerk

<table>
<thead>
<tr>
<th>Clinical evaluation</th>
<th>Score</th>
</tr>
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<tbody>
<tr>
<td>Continuous brandy-</td>
<td>2</td>
</tr>
<tr>
<td>hopping gait</td>
<td></td>
</tr>
<tr>
<td>Increased severity</td>
<td>3</td>
</tr>
<tr>
<td>Significant foot/</td>
<td></td>
</tr>
<tr>
<td>abnormal movement</td>
<td></td>
</tr>
<tr>
<td>Unable to control</td>
<td>1</td>
</tr>
<tr>
<td>maintain direction</td>
<td></td>
</tr>
<tr>
<td>(Inability to lift</td>
<td></td>
</tr>
<tr>
<td>stationary objects)</td>
<td></td>
</tr>
<tr>
<td>Body rolling, wide</td>
<td>2</td>
</tr>
<tr>
<td>based gait</td>
<td></td>
</tr>
<tr>
<td>Frequent stumbling</td>
<td></td>
</tr>
<tr>
<td>without falling</td>
<td></td>
</tr>
<tr>
<td>Falling</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Clinical mixture</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.7. Laboratory Analysis

Venous blood samples collected for biochemical analysis and haematocrit (PCV), and urine for USG, at 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 period and, 3) on day of necropsy. Additional venous blood samples were occasionally collected for analysis of serum bronide (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 21 G needles and 10 mL Vacutainer® tube 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 1000 rpm 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 StopFlow™ 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°C prior to removal to the laboratory for processing. Urine specific gravity (USG) was determined using a refractometer (VQ5000 refractometer, Vivaquip, Australia).

Haematological and biochemical analysis was performed by the laboratory within 60 minutes of blood collection. Haematological analyses were measured using a Celflex 3700 Haematology System (Abbott Diagnostics, Abbott Park, III, USA). Biochemical analysis was performed using an A&D FL400 clinical chemistry analyser (Thermo Electron Corp, Vantaa, Finland), using reagents from Thermo Scientific. See Appendix 1 for a full list of haematological analyses reported.

1.8. Necropsy

A full clinical examination was performed prior to euthanasia. At euthanasia, 100 U of heparin (Protex Heparin Pty Ltd) was injected into the jugular vein, followed slowly by Lethabarb (20 ml/40 kg bw). Immediately after euthanasia, the cranial articulation of C1 was exposed and cerebrospinal fluid (CSF) collected using a 23 g needle. The head was then removed and subjected to perfusion fixation. Briefly, the central arteries were exposed and a small catheter inserted and ligated to secure the perfusion line. Two litres of 0.15% phosphate buffered saline (PBS) containing 3000 IU of heparin/l was then slowly perfused via the ligated vessels at a pressure of 80 mm Hg using a Micronix peristaltic Pump (Micronix, Laguna Hills, Calif.). Once complete, catheters were removed and the whole head placed at 4°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.

Comparative fixation perfusion of the head, a routine ovine necropsy was performed and all general tissues taken for routine histopathology. These were: heart, lung, oesophagus, liver, spleen, pancreas, kidney, small and large intestine, bladder, seminal vesicles, testes and prostate (Shand Gray & Johnson, 1988). Tissues were fixed in formalin (10%) for 48 hours, dehydrated through grades of alcohol, cleared in xylene and embedded in paraffin wax. Sections (4–5 μm) were cut and stained with haematoxylin and eosin (H&E) using an automated staining system (Shandon Varanum Gemini 18 Slide Stainer, Thermo Fisher Scientific).

US 9,913,858 B2

19. Analysis of Serum and Cerebrospinal Fluid Contents

Serum bromide concentrations were determined by colorimetric spectrophotometry as previously described (Tierz 1970), with some modification. Briefly, 0.5 ml of serum was added to 4.5 ml of 10% trichloroacetic acid (Sigma-Alrich) in a 10 ml centrifuge tube, vortexed, then centrifuged for 15 min at 2000 g. 0.5 ml of supernatant was then mixed with 0.25 ml of 0.5% AuCl3 (Sigma-Alrich) 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. The lower limit of quantification (LOQ) was 25 μg/ml.

1.10. 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 1955), 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 >0.05 was the criterion for describing the data as a normal distribution. For analysis of biochemical data, a standard linear regression model was used where model assumptions were met. Spearman or Kendall’s correlation analysis was used for all other data sets.

Results

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 spinovisceral cardiovascular signs noted including eye deviation (Mayhew 2000). The movement disorder associated with ‘ryegrass staggers’ represents a specific sequence of dyskinesia (Combs, Rendell et al. 2014). To determine establishment of a model, a syndrome was 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 2.5).

2. Time to Onset and Clinical Signs Associated with Experimental Lofoten B Toxicosis

Neurological signs of lofoten B intoxication followed a clear progression. The first observable clinical signs were a fine tremor of the head and neck, stasis presenting as an alteration in stance or limb placement at rest which generally coincided with onset of Type 1 gait changes. Ventral ataxia 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. Observations of Type 2 gait changes were usually coincident within 4 days of falling. Menace reflex was either lost or reduced in lofoten B intoxicated animals but their papillary light reflex was normal.
Chapter 10.5, Appendix 5: Patent for treatment of toxicosis in sheep

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 at forelimbs, gross limbs, and/or body tremor. Inappropriate reaction during the movement, 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 stumps or lateral rostrum, generalized myoclonus, limb extension and opisthotonos, significant altered body stance at rest, heightened reactivity to noise movement, loss of menace reflex.

Histopathological changes at necropsy comprised those observed in case studies (Turner K, 2006, Cambridge, England, et al. 2014) with some additional features (see T10). Lesions were restricted to the caudal cerebellum and consisted of the specific histological features:

1. Spheroids (axonal swellings) located in the granule cell layer;
2. Presence of pyrulate granule cell neurons in the granular layer and less frequently;
3. Interalternation 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 represent a background lesion. Interalternation of Purkinje neurons is likely to represent the earliest neuropathological changes in this cell type. Interalternation of spheroids, in the absence of other notable pathology, is commonly reported a diagnostic lesion in cases of PSE or G1.

Lesions were observed in the highest number in those animals exposed to DL2 toxin only (Group 2, Table 3) with 5/9 animals presenting in the granular layer with spheroids and 6/9 exhibiting pyknotic cell bodies. A similar pattern was observed in the other two toxin treated groups (Groups 3 & 4, Table 4). In all toxin treated groups, spheroids and pyknotic cell bodies in the granular layer were the most common, while lower incidence of spheroids in the molecular layer was noted. The incidence included both the prophylactic treatment only group (Group 2) and control group (Group 1) suggesting that this may be an incidental finding in these cases (see Table 4).
Chapter 10.5, Appendix 5: Patent for treatment of toxicosis in sheep

US 9,913,858 B2

25 3) and lymphadenitis (animal 2, Group 5). Multifocal myo-

26 sis and multifocal lymphocytic myocarditis was observed in

all animals secondary to presence of serosysis. Again, these were suggested to be incidental background lesions

identified due to the size of the cohort.

3. Treatment with a Single Acute Dose of Oral Potassium Bromide Decreases Severity of Toxicosis, Increases Time to Falling and Improves Gait in Lolliten 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 500 mg/kg potassium bromide orally; time delay 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 is 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: 59.93 μg/ml; n = 5), falling slightly over the 48 hour treatment period to a final mean serum concentration of 104.54 μg/ml; n = 4, after 48 hours. This analysis indicates that high levels of bio-

availability of potassium bromide from 6 hours post treat-

ment (see Fig. 9). This data is supported by PK studies per formed prior to this trial in which high levels of bio-

availability were also observed with serum bromide levels peaking at 6 hours post administration (Combs and Edwards; personal communication).

Animals treated with a single oral dose of bromide 300 mg/kg. PK showed significant extension in time to falling 24 hours after treatment (Group 2 Day 1: 52.44 seconds; Group 3 Day 2: 145.66±22.67 seconds; students t test; p = 0.002, FIG. 10). Although no significant difference was observed between days 2 and 3 within treatment groups, differences between groups on both days were highly sig-

nificant (Group 2 Day 2: 145.66±22.67 seconds; Group 3 Day 2: 59.0±23.50 seconds; p = 0.003; Group 2 Day 3: 124.79±22.06 seconds; Group 3 Day 3: 51.52±23.23 sec-

onds; p = 0.002; FIG. 10). This data represents a significant increase in time to falling to Group 3 (toxin plus acute treatment) on days 2 and 3, compared to day 1, whilst a concomitant reduction in time to falling is observed in untreated animals (Group 1) (FIG. 10). 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 Fig. 8). A signifi-

cant 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 Lolliten B toxin and treated orally with 300 mg/kg LW potassium bromide (p = 0.001, FIG. 10). This is consistent with the improvement in maintenance of normal ambulation observed by increased time to falling post treatment (see Fig. 10). Conversely, composite gait scores are observed to increase in untreated intoxicated animals (Group 2) over the same time course (p = 0.001) showing a deterioration in gait over time.

15 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-treatment Day 1 of testing 5 (p = 0.002). Neither group showed a significant difference within groups between Days 1 and 3. However when Day 1: Day 3 tremor intensity rates are compared between groups there is a significant difference (p = 0.01, see FIG. 12). The median ratio for the Group 2 was 2.09 indicating an increas-

ing intensity of tremor whereas in Group 3 the mean ratio is 0.81 indicating a stabilisation of the tremor despite increasing intoxication over this time period (FIG. 12).

4. Serum and CNS Bromide Levels are Increased in the Presence of Lolliten B Intoxication

To determine whether animals were maintaining prophyl-

actic levels of bromide sufficient to be clinically effective after 22 days of administration, serum and CNS bromide concentrations were analysed on day of post mortem for all bromide treatment groups (Group 4: acute treatment 48 hours previously; 4: bromide prophylactic treatment plus lolliten B toxin for 22 days; 5: bromide prophylactic treatment only for 22 days). Serum and CNS concentrations of bromide were found to be significantly higher in the bromide prophylactic treatment plus lolliten B group 4 (Group 4) than in the prophylactic treatment group 5 alone (see FIG. 13) by a factor of 1.2:1 in serum and 3.7:1 in CNS. Data suggest that animals intoxicated with lolliten B in this study failed to excrete bromide with the same efficiency as their un intoxicated counterparts thus maintain-

ing higher levels of circulating bromide. The mean ratio of serum bromide:CNS bromide was also considered. This value was 0.74 for bromide prophylactic controls (Group 5); 0.91 for bromide prophylactic treatment plus lolliten B animals (Group 4) and 0.81 for acute bromide treatment plus lolliten B animals (Group 4). This suggests that intoxicated animals treated with bromide might also maintain higher bromide levels in CNS 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 falling and intox-

ication.

To investigate a possible mechanism for this difference, trace element copper (Cu) and zinc (Zn) levels was determined (DPI NSW Environmental Laboratory, Wollongbar, NSW). A very low level of total copper was found to be present in the serum and CNS suggesting that copper may be involved in the toxicosis. This data suggests that intoxicated animals treated with bromide might also maintain higher bromide levels in CNS 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 falling and intox-

ication.

5. Treatment with Potassium Bromide does not Aver

Prevalence of Neurological Lesions Observed in Experimen-

tal Cases of Lolliten B Intoxication

Identification of early lesions associated with clinical presentation of experimental bromism, and the 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 neurones of the Purkinje layer and granule cells. These lesions were first observed in the granule 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 FIG. 8). 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. These data suggest that treatment with potassium bromide does not mitigate underlying neuropathological cell damage associated with lithium toxicity despite alleviating some of the clinical signs of toxicity. Thus, the mode of action of the KBr treatment is not yet known.

Mild changes were noted in animals from Group 5 (bronze prophylactic treatment only) where one animal showed signs of a fine tremor and 4 animals were observed to show mild alterations in stance such as abnor-

mal foot placement at rest. Analgesia was also noted in 319 animals in this group such that they were more placid and easy to handle than their Group 1 counterparts and on occasion had to be encouraged to rise from rest in their pens. No overt neurologic signs were observed in any animal within the control group (Group 1). In those animals pre-

senting with neurologic signs associated with lithium toxicity, 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 those animals (see Table 4). Changes in body position (posture) 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 counter-

parts (Groups 2 and 3, Table 3). A marginal increase was observed in the number of animals exhibiting tremor 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 3).

The findings in this study represent a significant break-

through in available treatment options for the neurological deficits associated with perinatal gyrase toxicity. The clinical improvement in those observed in this study and the ability to abate tremor without sedation on delivery of an acute oral dose of potassium bromide are unique character-

istics of this therapy. It is one of administration and long half-life in the animal make it an ideal therapeutic interven-

tion for this plant toxicity.

REFERENCES


Chirn, M., B. Read, K. Steel, W. Mac and J. Quinn (2014). Evidence of dehydration and electrolyte distur-

bances in cases of perinatal gyrase toxicity in Austra-


The invention claimed is:

1. A method of preparing an animal for grazing in a selected pasture to prevent symptoms of litheness toxicity from developing in the animal during, or at completion of, grazing in the pasture, including administering a formulation

2. Administering an animal for grazing in a selected pasture in an amount effective for preventing the animal from developing symptoms of lithium toxicity before release of the animal to the pasture for grazing, wherein the formulation is administered to the animal orally,

3. Intravenously or intraperitoneally, and wherein the formulation includes bromide at a concentration of about 5 to about 70% w/w.

2. The method of claim 1, wherein the feed intake of the animal up to the time of administration of the formulation

3. The method of claim 1, wherein the animal does not suffer grass toxicity at the time of administration of the formulation.

4. The method of claim 1, wherein the animal does not suffer a movement disorder at the time of administration of the formulation.

5. The method of claim 1, wherein the animal is a ruminant.

6. The method of claim 5, wherein the ruminant is ovine or bovine.

7. The method of claim 1, wherein the formulation provides bromide in an amount of about 10 to about 750 mg/kg.

8. The method of claim 1, wherein the formulation is administered once daily for a period of one day to no more than about four weeks prior to releasing the animal to the selected pasture for grazing.

9. The method of claim 1 including the further step of administering a formulation including bromide to the animal

10. After the animal has been released to the selected pasture for grazing.

11. The method of claim 9, wherein the formulation is administered for a period of one day to no more than two weeks from release of the animal to the selected pasture for grazing.

12. The method of claim 11, wherein the bromide is in the form of potassium bromide or magnesium bromide.
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10.6 APPENDIX 6: PATENT FOR TREATMENT OF STRESS IN GRAZING ANIMALS

(12) United States Patent
Quinn et al.

(10) Patent No.: US 10,271,566 B2
(45) Date of Patent: Apr. 30, 2019

(54) STRESS MANAGEMENT IN LIVESTOCK

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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§ 371 (c)(1), (2) Date: May 20, 2016

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PCT Pub. Date: May 28, 2015

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A23K 50/10 (2016.01)
A61K 9/00 (2006.01)

(52) U.S. Cl.
CPC .......................... A23K 20/24 (2016.05); A23K 20/20 (2016.05); A23K 20/22 (2016.05); A23K 50/10 (2016.05); A61K 9/0095 (2013.01); A61K 33/00 (2013.01)

(58) Field of Classification Search
CPC .......................... A23K 50/10
See application file for complete search history.

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(74) Attorney, Agent, or Firm — Fitch, Even, Tabin & Flannery, LLP

(57) ABSTRACT
A method for minimizing or preventing the induction of stress-related or stress-induced inaptitude or inattention in an animal selected for a marketing or management practice.

17 Claims, 9 Drawing Sheets
Figure 1

Freezing Episodes: Novel Arena

Treatment

Control  Bromide treatment

Freezing Response Episodes

0  20  40  60  80  100
Figure 2

Parallel Rod Touches

0 20 40 60 80
Contro Bromide treatment
Figure 3

Weight gain in bromide treated mice

Weight Change (g)

Control  Bromide

-1.0  0.0  0.5  1.0  1.5  2.0

*
Figure 5

![Graph showing serum concentration levels over time](image-url)
Figure 6
Figure 7

Bromide treated versus untreated mulesed lambs
weight gain
over 51 days post treatment

Kg

Control
Bromide

Days post treatment
Figure 8

Bromide treated versus untreated mulesed lambs
weight change
over 51 days post treatment

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<tr>
<td>Day 51</td>
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</tbody>
</table>

- Control
- Bromide
Figure 9

Bromide treated versus untreated lambs weight Kg/h/day weight gain post mulesing

- Control
- Bromide
Chapter 10.6, Appendix 6: Patent for Treatment of Stress in Grazing Animals

US 10,271,566 B2

1

STRESS MANAGEMENT IN LIVESTOCK

CROSS-REFERENCE TO RELATED APPLICATION

This application is a U.S. national phase application of International Application No. PCT/US2014/050362, filed Nov. 20, 2014, designating the United States, which claims priority to Australian Patent Application No. 2013904516, filed Nov. 20, 2013, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

This invention relates to prevention of stress in livestock, especially ruminant animals.

BACKGROUND OF THE INVENTION

Many of the management practices to which livestock and ruminant animals are subjected can be potent physiological and psychological stresses that impact on the performance of the animal. Common-practice husbandry practices including tail docking, marking, castration, castration and dehoring are of particular concern, as are a number of other management practices including worming, transport, feedlot integration and other integration practices leading to mixing of unfamiliar animals, release of animals into an unfamiliar environment or into confined conditions, or presentation with unfamiliar feeds.

One manifestation of these stressors is inattenuation, otherwise known as exhaustion resulting from lack of nourishment. In particular, ruminant animals, which are highly susceptible to development of stress-induced conditions such as anemia, tend to reduce their nutrient intake (resulting in inattenuation), and leading to inattenuation and reduced growth performance and productivity. This is particularly seen in transport of live animals, especially in live export, and in the introduction of animals into feedlots for finishing.

There are a number of approaches to addressing this problem. Many of these have generally focused on treatment of animals that have been exposed to stressors, and with no particular focus on preparing an animal prior to implementation of a management practice so that, as prepared, the animal is then less susceptible to the stressors arising from, or associated with, the relevant practice.

There remains a need to minimise stress-induced inattenuation in livestock production (for example by minimising, or reducing the likelihood of, inattenuation), and in particular in the management and marketing of ruminant animals.

There is a particular need to minimise stress-induced inattenuation in animals selected for transport, including transport for other than slaughter purposes, and including live export.

There is also a need to minimise stress-induced inattenuation in animals selected for feedlot integration.

Reference to any prior art in the specification is not an acknowledgment or suggestion that this prior art forms part of the common general knowledge in any jurisdiction or that this prior art could reasonably be expected to be understood, regarded as relevant, and/or combined with other pieces of prior art by a skilled person in the art.

SUMMARY OF THE INVENTION

The invention seeks to address one or more of the above mentioned problems or needs, and in one embodiment provides for the treatment or preparation of an animal. The preparation of the animal is implemented prior to subjecting the animal to a marketing or management practice. This prior implementation minimises or prevents the induction of stress or anxiety-related inattenuation in the animal during, or at completion of, the practice. The preparation involves the administration of a formulation including bromide. The bromide is provided in an amount effective for preventing the animal from reducing feed intake, or more generally, from exhibiting reduced growth or productivity from lack of nourishment.

In one embodiment, the invention provides a method for the preparation of an animal prior to subjecting the animal to a marketing or management practice, thereby minimising or preventing the induction of stress or anxiety-related inattenuation in the animal during, or at completion of, the practice, the method including the step of administering a formulation including bromide to an animal selected for the marketing or management practice in an amount effective for preventing the animal from reducing feed intake.

In one embodiment, the invention provides a method for preparation of an animal for a marketing or management practice, thereby minimising or preventing the induction of stress or anxiety-related inattenuation in the animal during, or at completion of, the practice, the method including the step of administering a formulation including bromide to an animal selected for the marketing or management practice in an amount effective for preventing the animal from reducing feed intake.

In certain embodiments, the invention provides a method for preparing an animal for a husbandry procedure so as to minimise the likelihood of the animal developing stress or anxiety-related inattenuation subsequent to the procedure, the method including:

- providing bromide to an animal that has been selected for a husbandry procedure in an amount sufficient for minimising the development of stress or anxiety-related inattenuation in the animal;
- thereby preparing the animal for transport.

In another embodiment, the invention provides a method for preparing an animal for transport so as to minimise the likelihood of the animal developing stress or anxiety-related inattenuation during or subsequent to transport, the method including:

- providing bromide to an animal that has been selected for transport in an amount sufficient for minimising the development of stress or anxiety-related inattenuation in the animal;
- thereby preparing the animal for transport.

In a further embodiment, the invention provides a method for preparing an animal for feedlot integration so as to minimise the likelihood of the animal developing stress or anxiety-related inattenuation subsequent to feedlot integration, the method including:

- providing bromide to an animal that has been selected for feedlot integration in an amount sufficient for minimising the development of stress or anxiety-related inattenuation in the animal;
- thereby preparing the animal for feedlot integration.

In the above described methods, in one embodiment, the animal to which the invention is applied does not have stress...
or anxiety-related inanition or inappetence at the time of administration of the formulation. For example, the feed intake or growth performance of the animal up to the time of administration of the formulation is generally considered to have been normal in the context of the relevant age, physiology and environment pertaining to the animal.

In further embodiments there are provided compositions and kits suitable for use in the above described methods. In one embodiment, the animal is a ruminant (e.g. ovine or bovine).

Further aspects of the present invention and further embodiments of the aspects described in the preceding paragraphs will become apparent from the following description, given by way of example and with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Freezing response episodes in C57Bl/6, 4 month old male mice given oral potassium bromide 250 mg/kg. (n=9) or normal drinking water (n=5) for 7 days prior to novel arena testing.

FIG. 2. Enrolled fad tachos in C57Bl/6, 4 month old male mice given oral potassium bromide 250 mg/kg. (n=9) or normal drinking water (n=5) for 7 days prior to testing.

FIG. 3. Weight gain in C57Bl/6, 4 month old male mice given oral potassium bromide 250 mg/kg. (n=5) or normal drinking water (n=5) for 7 days under mildly stressful management conditions.

FIG. 4. Serum concentrations (menaNSD) of bromide after intravenous administration to eight sheep at a dose of 120 mg/kg.

FIG. 5. Serum concentrations (menaNSD) of bromide after oral administration to eight sheep at a dose of 120 mg/kg.

FIG. 6. Merino ewe lamb-bather mulesing and tail docking procedure.

FIG. 7. Merino ewe lambs mean weight gain over time after mulesing and tail docking (bromide animals received 300 mg/kg L-1 oral potassium bromide immediately prior to the procedure; n=20 in both control and bromide groups).

FIG. 8. Merino ewe lambs mean weight change over time after mulesing and tail docking (bromide animals received 300 mg/kg L-1 oral potassium bromide, as the potassium salt, immediately prior to the procedure; n=20 in both control and bromide groups).

FIG. 9. Merino ewe lamb mean weight gain per head/day after mulesing and tail docking (n=20 in both control and bromide groups).

DETAILED DESCRIPTION OF THE EMBODIMENTS

As described herein, the inventors have found that bromide may be used prophylactically to prevent an animal from developing stress-induced or stress-related inanition or inappetence to improve their health and well-being. This is accomplished by administering the bromide to the animal prior to subjecting the animal to a marketing or management practice, thereby minimising or preventing the induction of stress or anxiety-related inanition in the animal during, or at completion of, the practice. The method includes the step of administering a formulation including bromide to an animal selected for the marketing or management practice in an amount effective for preventing the animal from reducing feed intake, or from exhibiting reduced growth or productivity from lack of nourishment.

According to the invention, the relevant stress is that generally arising from farming practices, including management and marketing practices that the animal is routinely subjected to. In some contexts the stress may be referred to as ‘ante mortem’ stress which is generally understood to be stress that occurs pre-slaughter, and also more broadly in across the animal production lifecycle. Examples of relevant stress include stress resulting from transport, holding, handling, confinement, changes in feed or environment, mixing with unfamiliar animals and other like management practices relevant to livestock animals. Stress arising from transport, feedlot integration and other integration practices leading to mixing of unfamiliar animals, or release of animals into an unfamiliar environment are of particular concern, as is stress arising from husbandry practices such as mulesing, tail docking, dehorning, castration and marking procedures, and any other act of veterinary science.

In one embodiment, the marketing or management practice is a husbandry practice.

In accordance with the invention, there is provided a method for preparing an animal for a husbandry practice so as to minimise the likelihood of the animal developing stress or anxiety-related inanition subsequent to, or during, the practice. The method includes:

(providing bromide to an animal that has been selected for a husbandry practice in an amount sufficient for minimising the development of stress or anxiety-related inanition in the animal;)

thereby preparing the animal for the husbandry practice.

In one embodiment, the method includes:

(providing bromide to an animal that has been selected for transport in an amount sufficient for minimising the likelihood of the animal developing stress or anxiety-related inanition during or subsequent to transport, the method including:

(providing bromide to an animal that has been selected for feedlot integration in an amount sufficient for minimising the likelihood of the animal developing stress or anxiety-related inanition subsequent to feedlot integration, the method including:

(providing bromide to an animal that has been selected for feedlot integration in an amount sufficient for minimising the development of stress or anxiety-related inanition in the animal; thereby preparing the animal for feedlot integration.
Typically an animal to which the invention is applied does not have stress or anxiety-related irritant or insufficiency at the time of administration of the formulation. For example, the feed intake or growth performance of the animal up to the time of administration of the formulation is generally considered to have been normal in the context of the relevant age, physiology and environment pertaining to the animal.

In one embodiment, the animal does not have a movement disorder such as an abnormal gait, or a disorder arising from gross toxins, at the time of administration of the formulation.

As used herein, the term "preparing" or "preparation" refers to the use of bromide in a prophylactic or preventative sense. That is, preparing an animal for a marketing or management practice involves administering bromide (or a formulation including bromide) to the animal before it is subjected to the marketing or management practice so as to minimize, or reduce, or prevent the stress or anxiety-related irritation that the animal would otherwise experience in the absence of said preparation. Successful preparation of the animal will be achieved when the animal's normal feed intake is not significantly reduced or affected, or where the animal does not become significantly excited, disabled, or does not exhibit reduced productivity from lack of nourishment. For example, the animal's growth performance will not be significantly affected (e.g. the animal's weight, reproductive performance and/or milk production will remain around normal levels or increase).

As used herein, the term "animal" refers to domestic ruminant and monogastric animals, including swine, horses, cattle, bison, sheep, don, mule, elk, carabos and goats, of any age.

In one embodiment, the animal is a ruminant. Preferred ruminants include cow (i.e. beef) and bovine (i.e. cattle). In one embodiment, the animal is not an equine. The invention is also not intended to cover humans. Therefore, in one embodiment, the animal is not a human.

As used herein, "bromide" refers to the bromine ion (Br\(^{-}\)). It will be understood by a person skilled in the art that bromide will generally be administered to the animal in the form of a salt and/or other compound with a bromine group that readily dissociates into its and/or in vivo to give bromide. Preferably, the bromide is in the form of a salt, which may contain an aluminum oxide or aluminum oxide earth metal. For example, the bromide may be in the form of potassium bromide, sodium bromide or magnesium bromide. Potassium bromide and magnesium bromide are particularly useful for oral administration and sodium bromide is useful for intestinal administration. Magnesium bromide has a higher percentage of bromide than both the potassium and sodium salts, and possesses much greater water solubility than both the potassium and sodium salts. Consequently, magnesium bromide solutions can be made at much higher bromide concentrations than other salts, before saturation is reached. This property of the magnesium salt of bromide impacts on formulation; low volume delivery is practically and economically advantageous.

In one embodiment, bromide is provided in an amount of about 10 to about 750 mg/kg animal weight per dose. For example, the bromide may be provided in an amount of from about 20 to about 600 mg/kg animal weight per dose (e.g. from about 50 to about 500 mg/kg animal weight, from about 50 to about 400 mg/kg animal weight or from about 100 mg/kg to about 500 mg/kg animal weight). In one embodiment, the bromide is provided in an amount of about 300 mg/kg animal weight per dose.

Bromide may be provided in an amount of about 10 mg/kg, about 20 mg/kg, about 50 mg/kg, about 75 mg/kg, about 100 mg/kg, about 120 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300 mg/kg, about 350 mg/kg, about 400 mg/kg, about 450 mg/kg, about 500 mg/kg, about 550 mg/kg, about 600 mg/kg, about 650 mg/kg, about 700 mg/kg or about 750 mg/kg per dose.

In one embodiment, the bromide (e.g. in a formulation including bromide) is administered in an amount to provide an animal with about 10 to about 500 mg/kg animal weight of bromide. For example, the bromide may be in an amount to provide the animal with from about 20 to about 600 mg/kg animal weight (e.g. from about 30 to about 500 mg/kg animal weight, or from about 50 to about 400 mg/kg animal weight) of bromide. In one embodiment, the bromide is in an amount to provide an animal with about 300 mg/kg animal weight of bromide.

Bromide (e.g. in a formulation including bromide) may be administered in an amount to provide an animal with about 10 mg/kg, about 20 mg/kg, about 50 mg/kg, about 75 mg/kg, about 100 mg/kg, about 120 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300 mg/kg, about 350 mg/kg, about 400 mg/kg, about 450 mg/kg, about 500 mg/kg, about 550 mg/kg, about 600 mg/kg, about 650 mg/kg, about 700 mg/kg or about 750 mg/kg animal weight of bromide.

It will be understood that the specific dose level of bromide for any particular animal may depend upon a variety of factors including the activity of the specific bromide employed, the age, body weight, general health, sex and/or diet of the animal, time of administration, route of administration, and rate of excretion, drug or supplement combination (i.e. other drugs or supplements being used concomitantly with the bromide), and the severity of the stress being exhibited, if being used as a treatment.

In one embodiment, bromide is the only active in the formulation. In one embodiment, bromide is the only active provided to the animal. Therefore, in one embodiment, the method of the present invention does not include providing therapeutic or nutritive agents, other than bromide, to the animal. Examples of such agents include electrolytes (e.g. sodium, potassium, magnesium, manganese, chromium, and calcium, and chloride, oxide, carbonate or sulfate salts thereof), amino acids (or salts thereof), and sources of energy (e.g. sugars). Typically the formulation is provided once daily for a period of one day to no more than about four weeks prior to the time the animal is subjected to the marketing or management practice. In one embodiment, the formulation is provided once prior to subjecting the animal to a marketing or management practice. The formulation may be provided immediately prior to subjecting the animal to a marketing or management practice, or may be provided from one to 24 hours prior to subjecting the animal to a marketing or management practice.

In another embodiment, bromide is not given on or after completion of the marketing or management practice. A dose of a bromide-containing formulation of the invention may be delivered at once, for example, as a bolus, or over the course of several hours. Theoretically there is no maximum limit for the dosage provided that the bromide does not acerate in the animal to a point whereby it diminishes the quality of the animal or products therefore.
Chapter 10.6, Appendix 6: Patent for Treatment of Stress in Grazing Animals

The bromide may be provided in the form of a formulation adapted for delivery by an animal handler, or for consumption by the animal. Examples are described further below.

In one embodiment, the invention provides a formulation for use in preventing the induction of stress or anxiety-related inanition in an animal during, or at completion of, a marketing or management practice on an animal selected for the practice, the formulation including bromide in an amount for preventing the induction of stress or anxiety-related inanition in the animal, the formulation for administration to the animal, prior to subjecting the animal to the practice, in any of the embodiments described in the specification.

In one embodiment, the method includes a further step of administering a formulation including bromide to the animal at completion of the marketing or management practice, or thereafter. It will be understood that in this embodiment the animal selected for the relevant procedure first receives the bromide formulation before the relevant procedure. At the completion of the procedure, the animal may then receive further bromide for a pre-determined period. In this embodiment, typically the formulation is provided daily for a period of one day to no more than four weeks from completion of the marketing or management practice.

The formulation may be provided in a range of forms depending on the route of administration required. For example, the formulation may be adapted for injection intravenously, intramuscularly or subcutaneously, for oral, topical, ocular and nasal delivery, or for anal and vaginal delivery. Preferably the formulation is administered orally or intravenously. Accordingly, preferably, the formulation is provided in the form of a drench, or in the form of an injectable composition adapted for intravenous injection of the formulation.

In one embodiment, the formulation includes, in addition to the bromide, one or more pharmaceutically-acceptable excipients, such as binders, disintegrants, dispersants, lubricants, colours, flavours, coatings, glibidants, sorbents, absorbent-holding agents, emulsifiers, surfactants, buffers, bulking agents, toxicity-adjusting agents, preservatives, wetting agents, solvents, and sweeteners. Suitable examples of excipients to include in the formulation for use in the invention are well-known to a person skilled in the art.

Suitable formulations for use in the present invention include drenches, gels, pastes, tablet/holus formulations, gelatin capsules, injectable formulations, or intramuscular devices described above. In one embodiment, the invention provides a drench including bromide, whereby the drench includes bromide in an amount to provide an animal with about 20 to about 500 mg bromide/kg animal weight of bromide. In one embodiment, the drench includes bromide in an amount to provide an animal with about 20 to about 400 mg/kg animal weight (e.g. from about 30 to about 300 mg/kg animal weight, or from about 50 to about 400 mg/kg animal weight) of bromide. In one embodiment, the drench includes bromide in an amount to provide an animal with about 50 to about 400 mg/kg animal weight of bromide.

In one embodiment, the drench includes bromide in an amount to provide an animal with about 10 mg/kg, about 20 mg/kg, about 50 mg/kg, about 75 mg/kg, about 100 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300 mg/kg, about 350 mg/kg, about 400 mg/kg, or about 500 mg/kg animal weight of bromide. Preferred drenches are those adapted for use in a ruminant animal, particularly sheep (ovine) or cattle (bovine). Preferably the concentration of bromide in the drench is from about 5 to about 70% w/w (liquid or paste formulation) and about 5 to about 70% w/w (powder or solid formulation).

In one embodiment, the concentration of bromide in the drench is from about 10 to about 60%, from about 20 to about 50%, or from about 30 to about 40% w/w or w/v. In one embodiment, the concentration of bromide in the drench is about 5, about 10, about 20, about 30, about 40, about 50, about 60 or about 70% w/w or w/v.

In one embodiment, the only active in the drench is bromide.

In one embodiment, the drench is adapted for use in a monogastric animal.

In another embodiment the invention provides an injectable formulation including bromide, wherein the formulation includes bromide in an amount to provide an animal with about 10 to about 500 mg/kg animal weight of bromide. In one embodiment, the injectable formulation includes bromide in an amount to provide an animal with about 20 to about 400 mg/kg animal weight (e.g. from about 30 to about 300 mg/kg animal weight, or from about 50 to about 400 mg/kg animal weight) of bromide. In one embodiment, the injectable formulation includes bromide in an amount to provide an animal with about 300 mg/kg animal weight of bromide.

In one embodiment, the injectable formulation includes bromide in an amount to provide an animal with about 10 mg/kg, about 20 mg/kg, about 50 mg/kg, about 75 mg/kg, about 100 mg/kg, about 120 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300 mg/kg, about 350 mg/kg, about 400 mg/kg, or about 500 mg/kg animal weight of bromide. Preferably the concentration of bromide in the drench is from about 5 to about 70% w/w (liquid or paste formulation) and about 5 to about 70% w/w (powder or solid formulation).

In one embodiment, the concentration of bromide in the drench is from about 10 to about 60%, from about 20 to about 50%, or from about 30 to about 40% w/w. In one embodiment, the concentration of bromide in the drench is about 5, about 10, about 20, about 30, about 40, about 50, about 60 or about 70% w/w or w/v.

In one embodiment, the only active in the drench is bromide.

In one embodiment, the drench is adapted for use in a monogastric animal.
Chapter 10.6, Appendix 6: Patent for Treatment of Stress in Grazing Animals

US 10,271,566 B2

9 anxiety-related inattention in the animal, the formulation for administration to the animal, prior to subjecting the animal to the practice.

In one embodiment, the invention provides a formulation when used in a method of preventing the induction of stress or anxiety-related inattention in an animal during, or at completion of a marketing or management practice on an animal selected for the practice, wherein the active ingredient is bromide and wherein the bromide is present in an amount for preventing the induction of stress or anxiety-related inattention in the animal, the formulation for administration to the animal, prior to subjecting the animal to the practice.

In one embodiment, the invention also provides a formulation having an active ingredient for use in a method of preventing the stress or anxiety-related inattention in an animal during, or at completion of, a marketing or management practice on an animal selected for the practice, wherein the active ingredient is bromide and wherein the bromide is present in an amount for preventing the induction of stress or anxiety-related inattention in the animal, the formulation for administration to the animal, prior to subjecting the animal to the practice.

In one embodiment, the invention also provides the use of a formulation containing bromide in preventing the induction of stress or anxiety-related inattention in an animal during, or at completion of, a marketing or management practice on an animal selected for the practice, wherein the active ingredient is bromide and wherein the bromide is present in an amount for preventing the induction of stress or anxiety-related inattention in an animal, the formulation for administration to the animal, prior to subjecting the animal to the practice. The bromide may be dissolved in the animal’s drinking water for the animal’s consumption. The bromide may be incorporated into animal feed as a means to administer the bromide to the animal.

For example, bromide may be provided in an amount of between about 0.01 and about 5% w/w dry matter. In one embodiment, the concentration of bromide in feed is about 0.01, about 0.1, about 0.2, about 0.5, about 1, about 2, about 3, about 4 or about 5% w/w dry matter. The bromide may be administered with another medication (e.g. an antibiotic), a growth promoter, a pH adjuster or incorporated into a mineral premix. Suitable amounts of bromide in this regard include between about 0.1 and about 60% w/w dry weight. For example, bromide may be provided in an amount of about 0.5 and about 40%, about 0.1 and about 20% w/w dry weight. In one embodiment, the concentration of bromide is about 0.1, about 0.2, about 0.5, about 1 about 2, about 3, about 4, about 5% w/w dry matter.

It will be evident that, given the variable consumption of solids and liquids by animals, the amount of bromide in a consumable composition as stated above is approximate and may be varied depending on the type of formulation (solid v. liquid), the solubility of the bromide, the body weight of the animal and the average solid and liquid intake of the animal. Supplements may be provided with curries, or may be formulated into feed with bunkers. Exemplary carriers include grain or grass by-products, such as oats, barley, wheat, canola, rice, sorghum, millet, corn, legumes and grasses.

25 Typically, an animal treated by the method of the invention disclosed herein retains a normal feed intake subsequent to the marketing or management practice. It may be possible to further modify that feed intake by providing an appetite stimulant to the animal, prior to, during or after the completion of the marketing or management practice.

Example 1—Bromide Decreases Stress-Induced Inattention in Stress Treated Rodents

Monitoring Stress and Anxiety Behaviours in Rodents

A model system was established to induce stress in rodents by repeated behavioural testing using the Any-Maze™ rodent behavioural monitoring system. To determine the effects of bromide on these animals, aged, sex and strain matched animals were treated for 7 days with 250 mg/L potassium bromide in drinking water and subjected to mild stresses such as handling and weighing. At the end of 7 days animals were subjected to a number of behavioural tests to assess level of stress. Weight gain was also analysed in the treatment group by recording body weight over the initial 7 day period.

Results: Animals treated with bromide showed a trend towards decreased freezing response episodes (a behavioural stress response) and increased weight gain over the trial period (7 days).

Conclusions and clinical relevance: These data support potassium bromide as an efficacious therapeutic treatment to alleviate stress related behaviours and increase appetite in animals suffering from stress.

Materials and Methods

Determination of a Mild Stress Model in Rodents

C57BL/6J male mice at 5 weeks of age were purchased from MAS Client Services, Monash University, Melbourne, Australia. Animals were weighed at the start of the test period and daily for the duration of testing. Potassium bromide was administered in drinking water to 5 animals for 7 days at 250 mg/L, 5 control animals received water alone.

Behavioural Testing

Animals were weighed 3 times during the experiment (7 days). To initiate a mildly stressful situation, animals were exposed to novel areas, parallel rod and tremor tests using the mouse AnyMaze™ system (AnyMaze™, Stoelting, U.S.A.).

Novel Areas

Animals were placed in the centre of an open arena which was unfamiliar to them and their rate and range of movement tracked by digital recognition software. Distance, speed and time spent immobile were analysed using the AnyMaze™ software. Results were compared statistically using SPSS™.

Parallel Rod

The parallel rod test analyses changes in rate and range of movement as well as coordination. Briefly, animals are placed into a cage at the bottom of which is a sketched grid. If the animal places a foot below the grid and onto the floor.
Chapter 10.6, Appendix 6: Patent for Treatment of Stress in Grazing Animals

US 10,271,566 B2

111 a circuit will be activated and this abnormal footfall registered as an event. AnyMote™ tracking software also monitors rate of movement and periods of immobility.

112 Test Regime

113 Animals were placed in a tremor sensor box and a clear lid placed on top of the box. Movement was monitored for 2 minutes and data recorded using PowerLab™, ADI Instruments, Australia.

114 All animals were subjected to the following test regime encompassing both behavioral and tremor testing over a defined time period (see Table 1).

115

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Weight recorded</td>
</tr>
<tr>
<td>30 (1), 60 (1)</td>
<td>Tactile test</td>
</tr>
<tr>
<td>120 (2)</td>
<td>Tactile test</td>
</tr>
<tr>
<td>180 (2)</td>
<td>Tactile test</td>
</tr>
<tr>
<td>180 (3), 180 (3)</td>
<td>Tactile test plus novel arena</td>
</tr>
<tr>
<td>210 (3), 210 (3)</td>
<td>Tactile test</td>
</tr>
<tr>
<td>240 (4)</td>
<td>Tactile test plus parallel rod</td>
</tr>
<tr>
<td>270 (4)</td>
<td>Tactile test</td>
</tr>
<tr>
<td>300 (5)</td>
<td>Tactile test</td>
</tr>
<tr>
<td>330 (5)</td>
<td>Tactile test</td>
</tr>
<tr>
<td>360 (6)</td>
<td>Tactile test</td>
</tr>
</tbody>
</table>

116 At the end of the test period animals were euthanized by barbiturate overdose prior to perfusion fixation with 4% paraformaldehyde for routine histopathology and immunohistochemistry and blood analysis of bromide levels (results not reported as part of this application).

117 Results

118 a) Novel Arena test: freezing response episodes

119 Number of freezing episodes while performing the novel arena test were measured as an indicator of stress. The trend was towards decreased freezing episodes in animals receiving treatment with potassium bromide (see Fig. 1).

120 b) Parallel rod touches

121 On the parallel rod test, mice had significantly more touches if they were receiving bromide treatment. Although increased touches may be exhibited with ataxia these animals show no signs of ataxia and increased touches likely represent reduced anxiety about the novel environment (see Fig. 2).

122 c) Weight gain

123 Under mild stress conditions bromide increased weight gain in male/mated animals as compared to control (see Fig. 3).

124 Conclusion

125 Oral potassium bromide causes significant weight gain due to decreased anxiety and subsequent appetite stimulation in animals undergoing stress-related management practices.

126 Example 2—Determination of Pharmacokinetics of Bromide in Sheep after Single Intravenous (IV) and Oral (PO) Doses; Suitability of Bromide for Use as a Therapeutic Agent to Prevent Stress Induced Cessation in Ruminant Animals

127 Procedure

128 Briefly, sixteen Merino sheep were randomly assigned to two treatment groups. The intravenous (IV) group were given 120 mg/kg bromide, as sodium bromide. The per os (oral—PO group) were given 120 mg/kg bromide, as potassium bromide. Serum bromide concentrations were determined by colorimetric spectrophotometry.

129 Results

130 In summary, after IV administration the maximum concentration (Cmax) was 822.11±59.61 mg/L, volume of distribution (Vd) was 0.26±0.01 L/kg and the clearance (Cl) was 0.36±0.255 mg/L/kg. After PO administration the Cmax was 453.84±43.37 mg/L and the time of maximum concentration (Tmax) was 108±125 h. The terminal half-life (t1/2) of bromide after IV and PO administration was 587.93±15.35 h and 346.72±24.05 h, respectively. The oral bioavailability (F) of bromide was 92%. No adverse reactions were noted during this study in either treatment group. The concentration versus time profiles exhibited secondary peaks, suggestive of gastrointestinal cyclic redistribution of the drug.

131 Conclusions and Clinical Relevance

132 When administered PO, bromide in sheep has a long half-life (t1/2) of approximately 14 days, with good bioavailability.

133 Full Materials and Methods

134 Animals

135 Sixteen sheep, weighing between 49.5 kg and 67 kg and with an average body condition score of >2 were randomly divided into equal number (IV and PO treatment groups). Animals were placed in individual feeding pens and were fed twice daily on a ration of oats and lupins as well as ad libitum hay and water. Estimated carbohydrate content for oats and lupins was 0.11% and 0.4% respectively.

136 Indwelling intravenous cannulae (Braun, Certo, Sphenoswiss 335, 16 gauge, 32 cm) were placed into the left jugular vein and secured with 2/0 polypropylene suture. A 25 cm long, 21 gauge, IV extension set (HID) TUTA, 25cm minimal volume IV extension set) was connected to the catheter hub and the line was then flushed. Sodium bromide (NaBr) (Sigma-Aldrich) and potassium bromide (KBr) (Sigma-Aldrich) solutions were prepared using sterile water. The prepared NaBr solution was then filtered through a microfilter (0.22μm MILLEX GP, Cork, Ireland). Sodium and potassium salts were administered to dose sheep with 120 mg/kg of Br (154.6 mg/kg NaBr or 178.8 mg/kg KBr). All serum concentrations are for Br.

137 Potassium bromide is the most readily available form of Br for oral therapy. Sodium bromide was used for the IV study because of cardiotoxicity associated with potassium. Both salts are equally dissolvable in solution therefore no PK differences were expected.

138 IV Bromide

139 NaBr solution was administered through the cephalic vein using a 21 gauge needle over a period of 1 minute. Sheep were restrained in a seated position. Blood samples were collected at 0, 1, 5, 10, 15, 20 and 30 min and then at 1, 2, 3, 4, 6, 8, 10, 12, 24 h. Samples thereafter were collected at 12 h intervals to 240 h then at 24 h intervals to 336 h. A final sample was taken at 528 h. For each sample, the initial 2 ml of blood collected was discarded and a sterile syringe used to withdraw 5 ml of blood which was then placed into a plain separator blood tube (Vacutainer; Greiner Bio-one). The cannula was flushed with 3 ml of 3% heparinised saline after each collection. Each blood sample was left to stand for 30 min before centrifugation at 2000 g for 5 min. Serum was harvested and stored at -20°C until analysis.

139 PO Bromide

140 KBr solution was administered via an oesophageal tube, then flushed with 500 ml water. Blood samples were
collected at 0, 1, 2, 3, 4, 6, 8, 10, 12, 24 h, then at 12 h intervals to 240 h, then at 24 h intervals to 336 h. A final sample was taken at 508 h. When collecting blood samples at 1 h through to 30 h, heparin was aspirated over the catheter of a closed, syringe, to determine if the high salt load affected renal motility.

Determination of Serum Bromide Concentrations
Serum bromide concentrations were determined by colorimetric spectrophotometry as previously described (Tietz, 1976), with some modification. Briefly, 0.35 ml of serum was added to 5.35 ml of 10% methylenamine acid (Sigma-Aldrich) in a 10 ml centrifuge tube, vortexed, then centrifuged for 15 min at 2,000 g. 2.5 ml of supernatant was then mixed with 0.25 ml of 0.5% AgNO₃ (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 (R²=0.9992). The lower limit of quantification was 25 µg/ml.

Pharmacokinetics
Maximum concentration (Cmax) of Br and time to Cmax (Tmax) were determined directly from the data. Other PK parameters were determined for each sheep by use of non-compartmental analysis with a commercial software program (Toptit 2.6, Giftor Fischer Verlag). Area under the curve (AUC(0,t)) and area under the first moment curve (AUMC(0,t)) were calculated by the linear trapezoidal rule (Gibaldi, 1982) the terminal elimination rate constant (λz) was calculated by means of log-linear regression.

Whereas parameters Cmax, Tpeak, and AUC (where bioavailability is not absolute) are expected to differ when given IV or PO, Tpeak should be the same, regardless of route of administration. A test of the hypothesis of no difference between the t1/2 population means was performed. All results are expressed as mean±standard deviation (SD).

All sheep in the PO group exhibited no discernible neurological effects. There was no observed alteration in rumen motility and animals continued to eat and drink. Assessment of any acute neurological effects correlating with peak Br concentrations following IV administration was difficult as the sheep were held in the sternal position throughout the initial 20 min, for ease of sampling. All IV sheep walked back to their individual pens and subjectively observers reported a mild tranquillizing effect for approximately 1-2 h post-injection.

The relevant non-compartmental pharmacokinetic parameters derived from this study are summarised in Table 2.

<table>
<thead>
<tr>
<th>Pharmacokinetic variable</th>
<th>Intravenous administration (mean ± SD)</th>
<th>Oral administration (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (ng/ml)</td>
<td>821.1 ± 93.05</td>
<td>453.86 ± 43.37</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>10.8 ± 2.46</td>
<td>10.6 ± 2.46</td>
</tr>
<tr>
<td>AUC(0,t) (ng/ml*h)</td>
<td>157218.8 ± 52811.35</td>
<td>149400.6 ± 23138.10</td>
</tr>
<tr>
<td>MRT(0-∞) (h)</td>
<td>584.5 ± 22.6</td>
<td>413.4 ± 310</td>
</tr>
<tr>
<td>Cl (mL/h/kg)</td>
<td>0.855 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>V1 (L/kg)</td>
<td>0.286 ± 0.031</td>
<td></td>
</tr>
<tr>
<td>V2 (L/kg)</td>
<td>0.305 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

The concentration-time profiles for IV PO serum bromide are shown in FIG. 4 and FIG. 5, respectively. The t1/2 of Br in sheep following PO administration was 14.4 d, the IV t1/2 was 16.2 d; however, the difference between groups was not statistically significant (T=0.7822, df=18, p=0.4666).

An interesting finding in this study was the numerous pronounced peaks in the PO concentration versus time curve following the initial absorption phase. These peaks approached the Cmax seen on day one, but occurred many days later (FIG. 5). Indeed, some sheep had Tmax value well beyond the initial PO absorption phase. Similar although smaller peaks were also seen with IV bromide (FIG. 4).

Study Conclusions
Bromide was absorbed rapidly following PO administration. Prior to this study, it was predicted that absorption might be delayed due to dilution, the rumen having an estimated volume of 10l/45 kg of live weight (Hitchfield et al., 1991). However, the Cmax in this study was similar to that measured in horses given a comparable oral Br dose (Raidal and Edwards, 2000). Ruminal epithelial cells express large conductance channels permeable to chloride (Stampell et al., 2009) and the rumen has a high chloride absorptive capacity even against the net electrochemical gradient for the ion [Debono and Phillips, 1958, Scott, 1979]. It is likely that these high conductance channels are also responsible for overcoming a relatively lower initial Br concentration in the ruminal/gastric fluid compared with monogastric species.

The t1/2 of Br in sheep, of approximately 14 d, is comparable with humans’ 12-1 d (Harden et al., 1969) and in the dog 12-24 d (Trepanier and Ishibashi, 1995b). In classical PO pharmacokinetic profiles of monogastric species secondary peaks are uncommon, and are usually a function of unusual drug metabolism, in particular enterohepatic recycling. Bromide, however, is not metabolised. Possible causative mechanisms may be related to absorption, distribution and elimination. A Br study conducted in horses, at a similar PO dose to that used in this study produced a profile without secondary peaks in serum concentration (Raidal and Edwards, 2008).

Secondary peaks were also seen following IV administration, albeit much less pronounced (FIG. 4). However, it is notable that the IV peaks do appear to occur at similar time points to where they are seen following PO administration.
Chapter 10.6, Appendix 6: Patent for Treatment of Stress in Grazing Animals

15 The mean bioavailability of Br in this trial was 92%, which is much higher than the estimated bioavailability 32-38% in the horse (Reid and Edwards, 2008) and 40% in the dog (Trepanier 1995). This greater bioavailability in the sheep is probably due to the prolonged rumen residence time of ingestion (Cunningham et al., 2009).

16 The $V_d$ value of 0.286L/kg at 0.331L/kg as estimated from the literature (Hornby et al., 1997). The calculated volume of distribution at pseudo-equilibrium ($V_{eq}$), when equilibrium with rumen fluid is assumed, was 0.597L/kg for IV and 0.483L/kg for PO administration. Therefore, these values are similar, so a correction as $V_{d}$ is a proportionality factor relating to the concentration during the kg-linear phase of drug elimination, from which $t_{1/2}$ is derived.

15 Volume of distribution is the parameter used to calculate the loading dose (LD) using the equation $LD = V_d \times C_{d0}$, where $C_{d0}$ is concentration steady state, or the effective concentration for a particular use (as determined by IVR studies). The $V_d$ value is most appropriate where bromide is to be given as a PO bolus, and $V_{eq}$ in circumstances where it is to be given over a few days.

16 Because of the long $t_{1/2}$ following PO administration, the serum Br concentration fluctuated within a narrow range (approximately 200-400mg/L) for 14 days. This $t_{1/2}$ has a two-fold difference between peak and trough values over a two week period outperforms great potential for therapeutic application, in particular prophylactic use.

15 The study dose of Br, 120mg/kg, resulted in sustained Br concentrations approximating 75% of the lower bound of the animal's physiological range (10-20mg/L) (Pedell and Fenner, 1995). Concentrations of Br which will prevent, improve appetite or abolish stress are unknown, although it would be assumed that in all, the most severe cases to be substantially less than those required to protect against gram mal seizures.

Several advantages of the Br have now been identified by this study: Br is cheap, easy to administer, has good bioavailability and a low toxicity.

References


Example 3 – Practice of the Invention in Feedlot Integration

Steers enter the feedlot at 250 to 400 kg live weight, heifers enter at 200 to 375 kg live weight. The cows spend the first two weeks in their own pen—this period is referred to as induction. As induction is undertaken to reduce the stress of crowding and environment transition, bromide will be administered for part or the entirety of the induction phase. Provision of bromide during induction phase may occur via a single injection or injection of bromide 5 to 70% w/w or w/v, or by injection of bromide in feed within the range of 0.01% to 5% w/w dry matter, the lower value for a light feeder eating a bromide ration for 14 days to deliver a low dose of bromide and the upper value for a heavy steer consuming all bromide in three days to deliver a relatively high dose of bromide. Injection of bromide in an antibiotic, growth promotant or mineral premix at 0.1 to 60% w/w is a practical method of administering bromide to a total dose of 10 to 500 mg/kg. After induction the beasts are moved into the main feedlot in which they remain for up to 100 days to be grown out.

Example 4 – Practice of the Invention in Animal Transport

Animals are dosed with 10 to 500 mg/kg bromide either at the farm immediately prior to trucking to port, or during the induction phase at the port prior to boarding. Bromide to be administered by single injection or drench of bromide 10-70% w/w or w/v, within 48 hours of marking (excluding the procedures of mulesing, castration, ear tagging, vaccination and tail docking).

Example 5 – Practice of the Invention in Mulesing

Lams are dosed with 10 to 500 mg/kg bromide, administered by single drench of bromide 5 to 70% w/w or w/v, within 48 hours of marking (excluding the procedures of mulesing, castration, ear tagging, vaccination and tail docking).

Example 6 – Practice of Live Export of Sheep

Sheep between the ages of one and three years and weighing between 40 and 65 kg are road transported to a registered premises. Herd sheep, including rams up to 110 kg are also transported uncountonably. If the premises has sheds then they stay a minimum of three days, if going onto pasture they stay a minimum of five days (excluding day of arrival at and day of departure). Sheep are to be dosed with 10 to 500 mg/kg bromide either at the farm immediately prior to trucking to port, or during the induction phase at the port.

265
Chapter 10.6, Appendix 6: Patent for Treatment of Stress in Grazing Animals

Prior to weaning, Bromide to be administered by single injection or one of three 10 to 70% w/w or w/v, or by inclusion of bromide in pellets at a concentration of 0.01 to 5% w/w dry matter for 3 to 5 days.

Example 7—Practice of Shearing Rams

Rams to be dosed with 10 to 500 mg/kg bromide, within 48 hours of shearing. Bromide to be administered by single injection or inclusion of bromide 20 to 70% w/w or w/v.

Example 8—Practice of Mulesing in Sheep

One of the most severe management procedures undertaken in sheep production systems worldwide is that of mulesing. Mulesing is a routine breech modification procedure carried out on hoofed Merino flocks where significant amounts of skin growing in the breech region of the animal are removed to reduce the incidence of breech flystrike (infection of tissues of the breech area by larval stages of the Australian sheep blowfly (Lucilia cuprina) later in life). Flystrike is estimated to cause losses to the Australian sheep industry of $280 million annually (2013 figures, see Australian Wool Innovation Limited 2014, and MacKinnon Project 2015) and represents one of the more adverse health states observed in sheep production systems in Australia, and is a significant ethical concern in its own right as it is a condition that will inevitably lead to a slow and painful demise of animals if left untreated. Mulesing is mainly advocated in Merino lambs which are to be retained in the flock for breeding (replacement ewe lambs or, more rarely, ram lambs) in order to limit losses from breech fly strike in the flock.

Mulesing is routinely carried out without prior anaesthesia or anaesthesia with a hypnotic agent. An example of the speed at which this procedure can be carried out is that of mulesing a flock in 20 hours. Mulesing is a routine practice in most sheep flocks, and is carried out in combination with other management procedures including tail docking and marking (removal of male testes). The acute and chronic pain caused by a surgical procedure without anaesthesia is arguably the most extreme stressor/impairs maximal stress in an animal. This study is a robust test of the effectiveness of mulesing in minimizing the negative effects of breech flystrike.

To determine whether treatment with bromide at time of mulesing could limit the negative stress effects routinely observed in Merino flocks in response to this significant management procedure, a cohort of ewe lambs was identified which were due to undergo mulesing and tail docking as part of routine flock husbandry practices. Ewe lambs were either treated with bromide (as the potassium salt) orally immediately prior to mulesing and tail docking, or were left untreated, and their weight gain over time compared.

Methodology

A study was undertaken in a self-replacing merino flock to establish the effect of treatment with oral bromide (300 mg/kg body weight (BW) on weight gain post-procedure in Merino ewe lambs undergoing mulesing and tail docking as part of routine flock management practices. A total of 40 ewe lambs of approximately six weeks of age were dosed with 300 mg/kg bw of oral bromide with 20 receiving 300 mg/kg bw of bromide orally at time of procedure. A control group of 20 received 300 mg/kg bw of potassium bromide orally at time of procedure.

Briefly, the lambs were subjected to mulesing with the breech region presented to the operator. A sharp pair of shearing shears was used to make swift incisions into the skin of the lambs, removing the tear-shaped area of skin from the breech on both sides of the tail. Each incision leaves an area of open flesh approximately 5 to 10 cm in width depending on the amount of loose skin removed. The tail of the animal is removed to the third phalangeal joint by incision with hot cautery/ing "gas" shears and skin above the tail also pared back to expose the underlying tissue.

Animals were weighed on entry to the trial, treated orally with potassium bromide solution and then immediately subjected to mulesing and tail docking. Tri-Sol+™ (Oxier Animal Health, Gordon, NSW, Australia) was administered post-docking/mulesing to all exposed areas of underlying muscle as per manufacturer’s instructions. A control cohort was subjected to the same procedures but without treatment. All lambs were then returned to the mob and allowed to mother-up before returning to their usual pasture. All lambs were weighed at 7, 14, 21, 28 and 51 days post procedure. Statistical analysis was carried out using SPSSE™.

Results

A total of 40 ewe lambs of approximately six weeks of age entered the trial. 20 animals were treated orally with 300 mg/kg bw of 1.3W bromide (as the potassium salt) immediately prior to mulesing and tail docking, and 20 remaining, untreated whilst subjected to the same procedure. An example of an animal which had just undergone mulesing and tail docking is shown in Figure 4.

No significant difference was observed in entry weights of treated and untreated groups to the trial (Fig. 1). Mean initial live weights: Control group 13.6±0.49 kg; bromide treatment group 13.59±0.58 kg). Animals treated with oral potassium bromide (300 mg/kg (BW) at time of mulesing and tail docking showed a trend towards increased weight gain compared to untreated counterparts over the duration of the trial (51 days). Mean weight gain after 51 days for each group was: control 7.52±0.41 kg and bromide treatment group 8.52±0.57 kg respectively (see Fig. 7). Mean live weight on day 51 of the trial was observed to be 20.9±0.75 kg for control animals and 22.7±0.86 kg for bromide treated animals. A univariate pairwise analysis giving a significance of 0.30 (F= 1.092) to this data as has been previously suggested. No significant difference was observed in both groups after seven days (see Fig. 7). After 14 days, however, all animals had recovered their entry weights with bromide treated animals beginning to show enhanced weight gain compared to their untreated counterparts.

Figures showing the weight gain of both groups over the trial period (Fig. 7). A trend was observed after 14 days, however, all animals had recovered their initial live weights with bromide treated animals beginning to show enhanced weight gain compared to their untreated counterparts. All ewe lamb growth rate data are shown in Table 5. No significant differences were observed in weight gain between the two groups. In Table 5, the mean weight gain for the two groups was 7.6±0.57 kg and 8.5±0.57 kg respectively (see Fig. 8). Bromide treated animals also showed a marginally increased weight gain in the following 7 days period with mean weight gain for the two groups being 15.3±0.21 kg and 14.1±0.30 kg respectively (see Fig. 8). Bromide treated animals also showed a marginally increased weight gain in the following 7 days period with mean weight gain for the two groups being 15.3±0.21 kg and 14.1±0.30 kg respectively (see Fig. 8).
Chapter 10.6, Appendix 6: Patent for Treatment of Stress in Grazing Animals

US 10,271,566 B2

19 mentally in weight gain over the duration of the trial equating to a weight gain per head per day of 0.57.

20 Overall, improvement in weight gain in bromide treated animals equated to a 11% increase in production gain over the duration of the trial (51 days—control) group live weight gain 9.14 kg per head per day; bromide treated group 10.16 kg per head per day).

25 These data equate to a measurable improvement in live weight gain after mulesing and tail docking in live weight gain in ewe lambs, particularly in the Merino breed. Early weight gain will equate to a final difference in adult weight over the same time period if animals are on the same plane of nutrition equating to a higher body condition score at time of joining which can directly translate into improved production outcomes overall.

References


It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

The invention claimed is

1. A method for the treatment or preparation of an animal or subjecting the animal to a marketing or management practice, thereby minimizing or preventing the induction of stress-related inanition or anxiety-related inanition in the animal during, or at completion of, the marketing or management practice,

the method including the step of administering a formulation including bromide to an animal selected for the marketing or management practice to subjecting the animal to a management or marketing practice in an amount effective for preventing the animal from reduc- ing food intake, wherein the animal does not have stress-related inanition or anxiety-related inanition at the time of administration of the formulation, and

administering a formulation including bromide to the animal at completion of the marketing or management

practice for a period of one day to no more than four weeks from completion of the marketing or management practice.

2. The method of claim 1, wherein the feed intake of the animal or the growth performance of the animal up to the time of administration of the formulation has been normal.

3. The method of claim 1, wherein the animal does not suffer gross toxicosis at the time of administration of the formulation.

4. The method of claim 1, wherein the animal does not have a movement disorder at the time of administration of the formulation.

5. The method of claim 1, wherein the animal is a ruminant animal.

6. The method of claim 5, wherein the ruminant animal is ovine or bovine.

7. The method of claim 1, wherein the marketing or management practice is a husbandry practice.

8. The method of claim 7, wherein the husbandry practice is mulesing, castration, tail docking or marking.

9. The method of claim 7, wherein the husbandry practice is dehorning.

10. The method of claim 1, wherein the marketing or management practice is animal transport.

11. The method of claim 1, wherein the marketing or management practice is introduction of the animal into a feeder.

12. The method of claim 11, wherein the introduction of the animal into a feeder is in conjunction with a practice selected from the group consisting of a husbandry practice, including mulesing, castration, tail docking, marking, or dehorning, and animal transport.

13. The method of claim 1, wherein the formulation provides bromide in an amount of about 10 to about 750 mg/kg animal weight.

14. The method of claim 1, wherein the formulation is provided once daily for a period of one day to no more than about four weeks prior to subjecting the animal to a marketing or management practice.

15. The method of claim 1, wherein the formulation is provided orally.

16. The method of claim 15, wherein the formulation is provided in the form of a drench.

17. The method of claim 15, wherein the formulation includes magnesium bromide or potassium bromide at a concentration of about 5 to about 70% w/w.

* * * *
Chapter 10.7, Appendix 7: Papers, Conference Proceeding and Poster Presentations related to Thesis

10.7 **APPENDIX 7: PAPERS, CONFERENCE PROCEEDING AND POSTER PRESENTATIONS RELATED TO THESIS:**

10.7.1 **2011 Poster Submission: Sheep CRC/ MLA Postgraduate Conference**

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**Perennial Ryegrass Toxicosis: Observations from the 2011 outbreak and future therapeutic options.**

Mr Martin Combs¹, Dr Scott Edwards², Mr David Rendell³, Dr Kevin Reed¹, Mr Tim Quast¹, Dr Jane Quinn⁴

¹ School of Animal and Veterinary Science, Charles Sturt University, NSW
² David Rendell and Associates, Hamilton, Victoria
³ Reed Pasture Science, Hamilton, VIC

**Perennial Ryegrass Toxicosis (PRGT)**

Perennial ryegrass is sown on over 6 million hectares of land in Australia. The majority (>80%) are old Australian ecotypes that are commonly infected with toxin-producing endophytic fungi. One of these toxins, lolitrem B, causes severe neurological deficits in animals ingesting infected grasses resulting in the clinical presentation known as PRGT or ‘ryegrass staggers’. Affected animals suffer from movement disorders, and in severe cases, seizures and death. Financial losses to the red meat industry as a result of PRGT were estimated to be $52.3MM in a 2006 MLA commissioned report but actual figures may be higher.

**Current findings from the 2011 PRGT outbreak in Victoria.**

During Autumn 2011 clinical investigations were undertaken during a peak of PRGT in grazing livestock around the Hamilton area of Victoria. Pasture samples from eight properties in the locality, as well as blood, feces, brain, spinal cord and adipose tissue were collected for analysis from 11 sheep and one calf. Three blood samples were also taken from two affected but ambulatory sheep and one sutram that had been recumbent for more than 24h but was deemed treatable.

**Clinico-pathological findings:**

- 92% of cases were dehydrated and 2 of 14 recumbent animals developed renal failure (Fig 2A).
- Both ambulatory sheep sampled were dehydrated and azotaemic.
- Three sheep, that had been recumbent for more than 24 hours, were treated with oral rehydration and made full recoveries (Fig 2B).
- Hyperkalaemia (39% of cases) and hypernatraemia (23% of cases) exacerbate muscle weakness, hypoferraemia, seizures and ataxia arising from lolitrem B ingestion increasing the likelihood of mortality in these cases.
- All recumbent animals had moderate elevations in CK, AST & fibrinogen indicative of generalised muscle damage and inflammation due to recumbency.

**Histopathological Findings:**

- Typical histopathological changes (Parkinje cell loss and axonal bodies in the cerebellar granular layer) were observed in some but not all clinical cases.
- One euthanised sheep was diagnosed with Listeriosis eliminating it from the study and reinforcing the value of a histopathological/toxidological diagnosis.

**Toxicological Findings:**

- Fat and laces produced consistent positive results for lolitrem B by HPLC analysis.
- Toxin analysis of serum samples was not useful, however the development of an ELISA assay may allow analysis of serum samples in the future.
- Lolitrem B levels in the animal samples did not correlate well with levels found in pasture samples from the same paddocks, this suggests plant selection may exacerbate toxicity.

**Pharmacological investigations:**

- Bromide has the potential to offset lolitrem B’s BK channel blockade through the non-selective hyperpolarization of cell membranes. Bromide is also relatively inexpensive, readily available to farmers and able to be administered orally through drinking water, supplemental food or feed.
- Pharmacokinetic studies undertaken indicate a high oral bioavailability and long serum half-life.

**Implications for future therapeutic interventions for PRGT:**

- Correlating animal tissue/feral/serum samples with pasture samples will enhance our understanding of disease aetiology in PRGT.
- This study identifies that dehydration and serum electrolyte abnormalities associated with PRGT may contribute to morbidity & mortality rates in affected flocks. It would be desirable to extend the testing done in this study to greater numbers of animals, particularly animals under heat stress.
- Development and communication of clear animal management guidelines for farmers will assist in reducing mortality rates.
- Sole agent therapies are unlikely to be effective without clear integrated management guidelines.
- Oral potassium bromide may be a useful therapeutic intervention for PRGT, having high bioavailability and long half-life in sheep.

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**Figures:**

- Fig 1. Sites visited during the Autumn 2011 outbreak of PRGT in south-west Victoria.
- Fig 2. PRGT affected sheep in Victoria, 2011. A) Lateral recumbency in a severely dehydrated PRGT case. B) Previously <24h recumbent animals treated with oral re-hydration.
- Fig 3. Parkinje axonal body, a typical finding in the sample group with severe disease.
- Fig 4. Mean serum [Br⁻] ± SD after IV/PO Br⁻.
10.7.2 Poster Submission: Australian Neuroscience Society 2013

Lolitrem B Intoxication Activates Neuronal Stress Pathways
M.D. Combs, G.E. Rogers, A.S. Hamlin, & J.C. Quinn

Introduction
Lolitrem B is an indole-diterpeneoid alkaloid toxin found primarily in perennial ryegrass and is a potent inhibitor of BK channels. Cerebellar ataxia, tremor and Parkinson's disease lesions have been extensively reported. In grazing livestock however it has been suggested that a more widespread syndrome exists including possible anxiety, hyperaesthesia, ataxia and cognitive dysfunction. This study used c-Fos up-regulation to determine the level of activation in neuronal stress pathways following acute lolitrem B exposure in the mouse. It was hypothesised that lolitrem B intoxication would activate neural pathways associated with stress, pain and hyperaesthesia in addition to prolonged induction of a tremor and cerebellar ataxia.

Methods
3 adult mice were injected with lolitrem B (2mg/kg, ip.) (n=4) or vehicle (50% DMSO) (n=4). Tremor analysis was performed 1 hour post injection using a pressure sensor and PowerLab™ (AD Instruments Pty) analyser. At 3 hours post injection mice were anaesthetised and transcardially perfused with 4% paraformaldehyde. Brains were sectioned at 40μm and c-Fos immunoreactivity was revealed using the avidin-biotin-horseradish peroxidase technique (1:5000, Santa Cruz Biotechnology, TX, USA). An additional cohort of mice was tested to characterise the period and severity of induced tremor. Tremor was analysed by selecting one minute epochs for Fast Fourier Transform (FFT) analysis. The ratio of power output at likely tremor frequencies (0.20Hz) to total power output (0.50Hz) was used to assess severity of tremor.

Results
Analysis confirmed a tremor in the range of 10-10Hz. Lolitrem B induced a tremor of prolonged duration. Induction of tremor is also accompanied by a marked decrease in voluntary movement.

Conclusion
Lolitrem B induced a pattern of c-Fos immunoreactivity that is consistent with previous clinical and experimental observations of anxiety, allodynia and hyperaesthesia seen with intoxication of grazing animals. This is the first time activation of these pathways has been directly associated with a BK channel blocker.

Micrograph showing c-Fos-positive labeling in the paraventricular hypothalamus (PVH), central amygdala (CeA), lateral preoptic area (LPO), median eminence (EME), paraventricular nucleus (PVN) and locus coeruleus (LC). 2mg/kg ip injection of lolitrem B dramatically increased the number of c-Fos-positive neurons in the PVN, CeA, LPO and EME. Scale bar = 200μm.

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Chapter 10.7, Appendix 7: Papers, Conference Proceeding and Poster Presentations

related to Thesis

10.7.3 Australian Neuroscience Society 2016

**Potassium Bromide Mitigates Clinical Signs of Intoxication Caused by the Indole Diterpenoid**

**Toxin Litoltem B.**

Martin A. Combs1 2, Scott Edwards2 3, Allan E. Kosell2, Adam Hamlin1, Joshua Schepens-Duivenvoorden3, Edward Narayan1, 2, and Jane C. Quinn1 2, 3

**Aims**

Litoltem B is an indole diterpenoid toxin found in perennial ryegrass. Although litoltem B confers beneficial effects to the plant, these are counteracted by adverse effects observed in animals that ingest it which can result in behavioural changes, ataxia, tremor and, in some cases, seizures and death (Tor-Agáyilbo et al. 2001). Mortality and morbidity of livestock can be in significant numbers (Cunningham 1999, Combs et al. 2014).

Currently no therapeutic treatment is available that can be delivered at a fast enough rate to mitigate the large-scale effects observed in animals during clinical outbreaks. Platforms for drug testing have been difficult to establish due to seasonal variations in litoltem B in pasture-based feeds and the difficulty in extracting the toxin from ryegrass (Findlay et al. 2007). To overcome this, a reproducible and reliable model of PRGT in sheep was established utilizing a feed containing high levels of litoltem B toxin. This model was compared to a robust model where animals were exposed to pure litoltem B toxin isolated from perennial ryegrass. Establishment of these model systems allowed a number of therapeutics to be trialed. The most significant improvements were observed in movement, behavior, and reduction of stress by administration of potassium bromide. Pharmacological products that could improve clinical outcomes, reduce morbidity and mortality can now be tested using this experimental model.

**Methods**

**Animals**

White Sussex x Marino first cross male lambs of between 10-12 months of age (n=27, live weight (LW) 36.1 ± 3.02 kg) were used. A feed containing toxic levels of litoltem B was generated by incorporation into a chaff diet of a novel endophyte-infested perennial ryegrass seed (GaA6 AR9, Grasslands Technology Ltd, New Zealand). All animals receiving toxic feed were exposed to a diet containing a final concentration litoltem B of 0.16 mg/kg LW q 24hrs. After 7 days acclimation, animals were exposed to feeds either + or − for Litoltem B. Any animal that did not show significant movement disorder at 21 days, toxin was increased to 0.27 mg/kg LW for the duration of the trial. Five treatment groups with each containing nine animals entered the trial: Group 1: − negative control; lusene chaff only; Group 2: − positive control; lusene chaff containing 0.16 mg/kg LW litoltem B; Group 3: − acute litoltem B treatment; lusene chaff containing 0.16 mg/kg LW litoltem B treated orally with 300mg/KG KBr (Sigma Aldrich) on first day of stopping drug regime; Group 4: prophylactic lusene treatment + lusene 0.16 mg/kg LW q 24hrs; Group 5: lusene control

4 groups of adult mice were treated as follows: 1) adult mice were injected with 50% Litoltem B + KBr or lusene control; 2) mice were injected with 50% Litoltem B + KBr or lusene control; 3) animals were injected with 50% Litoltem B + KBr or lusene control; 4) animals were injected with 50% Litoltem B + KBr or lusene control.

**Gait analysis**

Animals underwent physical and observational examination daily. Gait analysis was performed on the trial and then every third day during the trial. To achieve this, animals were circled in the yards for a minimum of three minutes whilst their movement was captured on video and a gait observation score determined using the following scoring criteria on a scale 0 (normal) to 5 (severe): ataxia, dyscoordinatedism, rhythmic myoclonus, stumbling or falling. Entry to the treatment phase of the study was when animals displayed sufficient dyscoordinatedism or rhythmic myoclonus to induce stumbling or falling. From point animals were assessed daily for gait changes for the subsequent 40 hours.

**Faecal Gastrocorticosteroid metabolite analysis**

Faecal cortisol metabolites (FCM) were extracted from sheep faecal samples using the methods as previously described for ruminants. FCM ELISA followed the detailed guidelines provided by Brown et al. (2003) for validating the FCM enzyme-immunoassay. Concentrations of FCM were determined using a polynomial antilogarithm (R468) diluted (115000:1), horse- radish peroxidase conjugated cortisol labelled diluent 1950000 and cortisol standards (1 50000:1g/ml). Samples were assayed (duplicate) on an immunoassay microplate reader (MAX-Scan II plates) (96) wells). Intra- and inter-assay coefficients of variation were determined from high concentration samples (25% of total area under the curve) included in all assays. Intra-assay coefficients of variation were 1.8% and 5.4% low and high percent control, and inter-assay coefficients of variation were 5.9% and 1.8% respectively.

**Tremor analysis on mice**

Tremor analysis was performed on mice using a piezoelectric pressure sensor and 2D instruments Pivotal10 software and Lutar10 software to characterize the period and severity of induced tremor. Tremor was analyzed by selecting one minute epochs for Fast Fourier Transform (FFT) analysis. The ratio of power output at likely tremor frequencies (6-20Hz) to total power output (0-200Hz) was used as a tremor ratio power ratio (MPTR) was used to assess severity of tremor.

**Results**

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

**Sheep Gait Analysis**

Following admission to the treatment stage of the protocol, animals given a single oral dose of bromide 300mg/KG UV showed reduced incidence of animals falling at 72 hours (Day 3) post treatment compared to untreated positive controls (Group 2) (Figure 1). They also showed improved composite gait scores (Figure 2) indicating that treatment with bromides improved both gait coordination and walking in this cohort. The majority of KBr treated animals did not exhibit falling during the 3 minute gait testing period on day 2 and 3 of the treatment protocol despite falling on Day 1. By comparison, their untreated counterparts showed greater incidence of falling and falling over the same time period. This data suggests improved coordination in the acute treatment group (Group 3) and observation with increasing time in their untreated counterparts (Figures 1).

**Faecal Cortisol Metabolites**

There was overall significant difference in mean levels of FCMs between the treatment groups (F = 2.854; p = 0.028; Figure 8). Post-hoc testing showed that mean FCMs levels were significantly different between groups 1 and 2. Also there was significant difference in mean FCM levels between groups 2 (positive control) and group 5 (treatment control). Overall mean levels of FCMs were as follows: Group 1 (8.86 ngg dry faecal mass), Group 2 (42.77 ngg dry faecal mass), Group 3 (8.16 ngg dry faecal mass) and Group 4 = 11.03 ngg dry faecal mass and Group 5 = 11.46 ngg dry faecal mass. There were no significant differences in mean FCM levels between group 1 with groups 3, 4, or 5 (p = 0.05 in all comparisons).

**Tremor Analysis on Mice**

Mice administered Litoltem B at 2mg/kg p.i. exhibited an increased MPTR when compared to litoltem B + bromide treated mice at all time points except for 0 and 1 hours. At 6 hours animals exhibited a tremor with bromide exhibited with significant difference in MPTR and control animals.

**References**

Chapter 10.7, Appendix 7: Papers, Conference Proceeding and Poster Presentations related to Thesis

10.7.4 Poster Presentation: Australian Sheep Vets Conference 2016

Treatment of Perennial Ryegrass Staggers with Potassium Bromide.

M Combs, A Hamlin, S Edwards, JC Quinn

Aims
Perennial Ryegrass Staggers (PRSS) is caused by ingestion of a mixture of toxins present in Lolium perenne and L. perenne var. perenne. These toxins cause significant mortality in sheep during severe outbreaks (Combs et al., 2012). Treatment of PRSS is based on the assumption that potassium is essential for the function of the potassium channel. The potassium channel plays a role in the influx of potassium ions into the cell membrane. The channel is known to be disrupted in PRSS, leading to the accumulation of potassium ions in the cell membrane. The potassium channel is a target for potassium channel blockers, which can be used to treat PRSS.

Methods
Animals
White Suffolk x Merino first cross rams sourced from between 10-12 months of age (mean = 27.1 kg) were used. The animals were randomly divided into two groups: a control group and a treatment group. The control group was injected with a saline solution, while the treatment group was injected with a potassium channel blocker.

Results
Treatment with a single acute dose of oral potassium bromide decreases severity of toxicity, increases time to falling and improves growth in lambs treated with potassium channel blockers.

Gut Analysis
Following treatment, the gut was examined and the potassium channel blocker was found to decrease the number of potassium ions in the gut wall. This decrease in potassium ions in the gut wall led to a decrease in the number of potassium ions in the cell membrane, leading to a decrease in the severity of toxicity.

Histopathology
Histopathological changes in the gut were examined and the potassium channel blocker was found to decrease the number of potassium ions in the gut wall. This decrease in potassium ions in the gut wall led to a decrease in the number of potassium ions in the cell membrane, leading to a decrease in the severity of toxicity.

Figure 1: Typical toxicity features in the gut wall of treated lambs. (A) Control group. (B) Treatment group. (C) Control group with potassium channel blocker. (D) Treatment group with potassium channel blocker.

Figure 2: Typical toxicity features in the gut wall of treated lambs. (A) Control group. (B) Treatment group. (C) Control group with potassium channel blocker. (D) Treatment group with potassium channel blocker.

Figure 3: Typical toxicity features in the gut wall of treated lambs. (A) Control group. (B) Treatment group. (C) Control group with potassium channel blocker. (D) Treatment group with potassium channel blocker.

New insights into the clinicopathological mechanisms and presentation of perennial ryegrass toxicosis in Australia.

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Abstract: Perennial Ryegrass Toxicosis (PRGT) is a common disease entity in Australia. Clinical signs of PRGT include abnormal behaviour, ataxia (‘staggers’), ill-thrift and gastrointestinal dysfunction (scours) (Cunningham 1959). PRGT is caused by toxins produced by the endophytic fungus Neotyphodium lolii, a symbiont of perennial ryegrass, that has widespread prevalence in pastures across south-east Australia and Tasmania (Gallagher, Campbell et al. 1982, Cheeke 1995). Clinical signs range in severity from mild gait abnormalities and failure to thrive, to severe seizures, lateral recumbency and death (Finnie, Windsor, Kessel, 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. 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 (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. During 2011, producers experienced a mild outbreak of PRGT across the state of Victoria that saw large numbers of affected animals associated with limited mortalities. Neurological observations identified two key gait abnormalities associated with intoxication and clinical samples taken from affected sheep showed dehydration and electrolyte abnormalities. These data suggest that changes in hydration status may be a contributory aetiological factor in years where high numbers of fatalities are associated with PRGT outbreaks in Australia.

Key words: perennial ryegrass; toxicosis; staggers; dehydration, lolitrem B; ergovaline.

Introduction

Perennial Rye Grass Toxicosis (PRGT) has a long history of prevalence in Australia and New Zealand. A disease of farm animals showing similar clinical signs to those now commonly
Chapter 10.7, Appendix 7: Papers, Conference Proceeding and Poster Presentations related to Thesis

Attributed to PRGT was first reported in 1880 (NZ Country Journal) with the first published report in the veterinary literature appearing in 1906 (Gilruth 1906). Gilruth described a condition in which muscular incoordination was associated with animals grazing ryegrass, particularly in summer and autumn. Since then the nature of the neurological derangement associated with PRGT has been better defined (Keogh 1973; Galey et al. 1993; Johnstone 2010) with Keogh (1973) providing a scale of clinical severity which has been commonly used in research to assess PRGT cases since its publication. In 1981, Lolitrem B, the indole diterpenoid toxin produced by the endophytic fungi Neotyphodium lolii, was first identified as the causal agent of the neurological signs associated with PRGT, a fact which is now widely accepted (Gallagher et al. 1981).

Lolitrem B is a potent inhibitor of calcium activated potassium (or BK) channels in the brain (Imlich et al. 2011) BK channel inhibition has been demonstrated to produce a form of ataxia by inhibition of Purkinje neurons of the cerebellum (Cavanagh et al. 1998). Purkinje cells are the major output neuron population of the cerebellum and synapses with neurons in the deep cerebellar nuclei which in turn synapse with various pre-motor centres in the brain to coordinate movement. In PRGT-affected livestock the extent of ataxia produced may therefore be determined by the extent of BK channel blockade in the brain with a mild blockade producing problems with pre-motor sequencing and initiation of movement and more severe blockade producing dystonia, myoclonus and "cerebellar seizures". One aim of this study was to see if clinical observations of gait and other neurological assessments could provide insight into the severity of clinical disease in field cases of PRGT.

In addition to motor disorders, PRGT-affected livestock exhibit other neurological signs including increased anxiety and allodynia (Johnstone 2010; Johnstone et al. 2012). BK channels are widely distributed throughout the central nervous system (Pwonska et al. 2008) and effects in other brain regions are likely to be causing these other neurological signs. BK channels are not only present in neural tissue but also in other organ systems in the body, in particular, vascular, renal, and gastrointestinal tissues (Smith et al. 1997; Plunzick and Sansom 2006; Wulf et al. 2009). A more widespread effect of Lolitrem B toxicity could therefore underlie other clinical signs associated with PRGT.

Despite the prevalence of PRGT, there is a paucity of published data on the clinicopathological changes found in animals affected with PRGT in the clinical setting. In this study we report clinicopathological findings from PRGT cases presenting during a mild PRGT outbreak in Victoria, Australia, in 2011. The study was undertaken during a peak of PRGT around the Hamilton area of Victoria. Pasture samples from eight properties in the locality, as well as blood, faeces, brain, cerebrospinal fluid, spinal cord and adipose tissue were collected from 13 sheep.
with clinical signs consistent with PRGT and ranging from ambulatory but ataxic through to those with prolonged recumbency and/or severe and continuing neurological signs of such severity that euthanasia was required. Producer interviews were conducted to better construct a global picture of PRGT on the properties visited with producers consulted regarding the extent of observed toxicosis (both with the current outbreak and over the past ten years), mitigation strategies used, any specific therapeutic agents administered to affected animals and measures implemented for both management of flocks and for severely affected individuals during the recent outbreak. Producers were also asked about their perceptions of production losses from PRGT.

Results

Clinical presentation of neurological signs associated with a mild PRGT outbreak.

Animals examined exhibited a wide range of clinical status; from those which were ambulatory and therefore exhibiting only mild to moderate intoxication to those that were severely affected, had been recumbent for a number of days and required euthanasia due to the severity of their clinical presentation. All animals examined exhibited a fine tremor of the facial muscles, neck and limbs. Tremor was more prominent in the fore than hind limbs. Recumbency reduced the severity of tremor but it could still be identified on careful examination and was exacerbated on any intentional movement. In the most severely affected cases, significant tremor was observed occasionally escalating to resemble generalised seizures. There was, however, no coincident loss of consciousness suggesting that these episodes were seizures of cerebellar origin.

A neurological examination was carried out on each animal. Cranial nerve function appeared normal and there was no apparent loss of menace reflex. Spinal reflexes were normal although mild hypo- or hyper-reflexia was observed. Sensation and withdrawal responses appeared normal. Conscious proprioceptive testing was undertaken in one ambulatory animal, E11 (Figure 1), and found to be normal despite significant tremor. The majority of recumbent animals examined attempted to take food when offered suggesting no severe cognitive deficit in these cases and lack of significant damage to those cranial nerves involved in prehension, mastication and swallowing. On one property, a number of sheep in sternal recumbency were observed to have crawled to graze despite being unable to rise independently or remain standing if assisted to do so. Animals that had been recumbent for longer periods of time (estimated to be 3 days or more) were visibly dehydrated and showed evidence of significant muscle wasting and anorexia (Figure 2).

Categorisation of severity of movement disorder in PRGT-affected sheep

Large numbers of mildly affected, ambulatory, animals were observed on all properties visited. These animals were found to exhibit two distinct types of gait abnormality, Type 1 and Type 2, which have been not been reported in detail previously:
Type 1 animals exhibited dysfunction in initiation of movement, a failure to correctly extend limbs in sequence as rate of movement increased; they showed mild head and neck tremor and often ended a period of movement with sudden collapse on either fore- or hindlimbs, usually into sternal recumbency. Type 1 animals were often observed to be unable to follow a desired direction of movement resulting in the animal failing to reach its goal (such as attempting to re-enter the flock).

Type 2 animals exhibited a shortened, bunny hopping gait with rigid limb hyperextension, typically falling into lateral recumbency on attempts at rapid movement. Immediately after falling the limbs would be rigidly extended, exhibiting a period of jerking and clonus, before the animal fell into lateral recumbency and limbs relaxed. Head and neck movements were usually normal with no extension of the neck observed suggesting myoclonus was predominantly in locomotor muscles. Both Type 1 and Type 2 animals could regain a standing position after a short period of recumbency.

Biochemical analysis of blood samples from PRGT affected animals.
Serum biochemistry analysis of venous blood samples revealed that all 13 sheep sampled exhibited clinical changes indicative of dehydration (Table 1). 12/13 cases showed serum albumin concentrations above normal range (hyperalbuminaemia) with the last animal exhibiting albumin levels at the top of normal range but with coincident azotaemia (elevated urea) (Table 1). Two recumbent animals showed evidence suggestive of renal failure secondary to severe dehydration despite a relatively short recumbency (animals 1 and 10). Electrolyte disturbances were present in a number of animals including hyperkalaemia (elevated potassium) (3/13) and hypernatraemia (elevated sodium) (3/13) (Table 1). Both of these electrolyte abnormalities have the potential to induce muscle weakness, hyporeflexia, and ataxia. In addition to electrolyte changes, most animals exhibited elevation in fibrinogen and creatine kinase indicative of muscle damage and inflammation (Table 1).

Histopathology of brain tissue from PRGT affected animals.
Histopathological findings were typical and consistent with those previously reported in clinical cases of PRGT with Parkinson's cell loss and axonal swellings in the cerebellar granular layer (Figure 3). In two cases small areas of haemorrhage within the cerebellum were also noted, this has also been found in histopathology examinations of other BK channel blockers such as penitrem A (Cavanagh et al. 1998). One sheep which presented on one of the affected properties, and had clinical signs consistent with PRGT (a case additional to those reported in Table 1) showed histopathological changes coincident with a diagnosis of listeriosis. This animal reinforces the value of histopathological diagnosis in cases of suspected PRGT.
Producer reported findings.

All properties visited had experienced outbreaks of PRGT previously and so were familiar with the clinical presentation in livestock. Pastures from which affected animals were taken contained predominantly perennial rye grass. The major long term mitigation strategy employed by the producers in this study was pasture renovation with novel endophyte strains of perennial rye grass or alternative pasture. This was usually done for selected “rescue” paddocks. Some producers report problems with regrowth of “old” perennial ryegrass. On one property, the producer had renovated selected paddocks with a novel endophyte perennial rye grass variety only to have animals affected by PRGT grazing the same paddocks in the following season. Producers typically did not report observations of clinical signs of PRGT in sheep to veterinarians, until a significant proportion of the flock showed severe movement abnormalities or significant mortalities was noted in the flock.

Mitigation strategies reported by producers after observation of PRGT in their flocks included reducing or abandoning routine animal husbandry procedures; reducing contact with affected animals to reduce stress; moving animals to a less or non-toxic pasture, and restricting access to open water sources. In one instance, the producer had moved a mob of sheep to a small containment paddock and fed them lucerne hay only. Some producers were providing animal access to toxin binding agents in the form of loose licks. In general, if the flock or mob could be moved without significant enhancement to clinical signs then this was achieved, if not then intervention for affected flocks was minimised and animals were allowed to run the course of their clinical signs.

Producer intervention for individual animals severely affected by PRGT varied greatly. Treatment of persistently recumbent individual animals was commonly minimal with animals often offered no treatment even after several days of recumbency. This appeared in large part to be due to the misapplication of advice for flock management to the individual animal. Farmers frequently cited that “stressing them makes them worse” and “animals are best left alone”. However there were notable exceptions and the producers who treated individual animals in prolonged recumbency (>4-12hours) typically reported a high success rate.

Discussion

Neurological changes, an indicator of level of intoxication in affected livestock.

This report presents a set of novel observation of the complex neurological changes observed in PRGT affected animals. Type 1 animals are those that appeared to have difficulty with rapid sequential movements and may be best defined as suffering from dysdiadokinesia. Dysdiadokinesia is a classic sign of cerebellar ataxia and suggests a reasonably focal effect on the
cerebellum and deep cerebellar nuclei (Diener and Dichgans 1992; Diener et al. 1993). Type 2 animals are those who, on attempting to move, progress to a rigid hyper-extended, bunny hopping gait, and may be better defined as presenting with rhythmic myoclonus. This presentation suggests a broader dysregulation of neurological activity with involvement of thalamic and brainstem nuclei.

The transition from Type 1 to Type 2 neurological signs is therefore likely to represent a progression in severity of intoxication in the animal and could be used as the basis of an observational model for judging the severity of a PRGT outbreak. As this severity scale uses discrete gait changes to classify severity of disease, it has some advantage over the Koegh scale which uses subjective estimates of degree of tremor to scale severity of PRGT. Researchers have also observed behavioural changes in sub-clinically affected animals, including hyperaesthesia, anxiety and absence of normal shade seeking behaviour, (Macintosh et al. 1982; Reed et al. 2011a; Johnstone et al. 2012). Codification of behavioural changes would extend the usefulness of any new observational scale to producers.

Evidence of dehydration in PRGT affected animals – a clue to the severity of clinical signs in Australian livestock?

Samples for this study were collected from animals presenting with PRGT during a ‘mild’ or atypical PRGT outbreak. During this outbreak, ambient temperatures were low and abundant green feed was widely available, this is not typical of outbreaks of PRGT in Australia where ambient temperatures are usually high and feed availability limited. However, despite the relative abundance of both feed and moisture, serum biochemical analysis of venous blood samples revealed that 12/13 sheep showed evidence of dehydration (Table 1) as defined by increased concentrations of serum albumin (Scott. 2008).

This is the first time evidence of dehydration has been reported in animals clinically affected by PRGT. Although elevation of albumin levels in the majority of animals examined in our study was not severe the trend is significant. Dehydration in our cohort was not related to ability to access water as the three ambulatory animals included in this study also showed albumin levels considerably higher than normal range (Table 1; animals 3, 11, 13) confirming dehydration in the absence of recumbency in these cases. Our finding of subclinical dehydration in ambulatory animals is supported by reports of increased water intake in animals exposed to perennial ryegrass pasture but not exhibiting clinical signs (Sewell et al. 2009).

Lolitrem B may play a direct role in the electrolyte changes observed in affected animals. Lolitrem B antagonises calcium-activated large conductance potassium channels (BK channels) (Imlach et al. 2011) and in the kidney and colon BK channels play a role in excretion of potassium,
particularly when levels are high (Holtzelaw et al. 2011). In BK knockout mice elevated levels of aldosterone compensate for the lack of functional BK channels, however it is unclear to what extent electrolyte homeostasis may be impaired in an animal with both BK channel blockade and dehydration (Rieg et al. 2007; Grimm et al. 2005a). Neotyphodium lolii also produces a range of toxic substances including the ergot alkaloid ergovaline (Cunningham et al. 1993). Co-toxicity, particularly lolitrem B-ergovaline toxicity, has been speculated as a compounding factor in PRGT cases (Tor-Agbidye et al. 2001; Reed et al. 2011) but, to date, has not been examined in a clinical setting nor has this interaction been proven in an on-farm situation (Reed et al. 2011). Ergovaline, could also play an important contributory role to exacerbating dehydration in PRGT cases. Ergovaline, as a dopamine D2 agonist, inhibits Angiotensin II-mediated release of aldosterone, therefore inhibiting its potassium excretion and water-sparing role in the kidney (Whitfield et al. 1980; Larson et al. 1995). It also causes peripheral vasoconstriction and, in high ambient temperatures, this leads to hyperthermia and increased respiratory tract water losses (Gadberry et al. 2003; Gooneratne et al. 2011). Transgenic mice lacking BK channels are known have decreased ACTH response to stress (Brunton et al. 2007), therefore Lolitrem B and ergovaline are likely to synergistically inhibit the excretion of cortisol, disrupting normal gastrointestinal function, a key mediatior of electrolyte exchange and fluid homeostasis in ruminants. How the effects of this combined toxicity exacerbated the clinical signs of PRGT in “on-farm” conditions is hard to determine but should be a key area for further investigation.

Producer intervention, a mixed approach to the management and treatment of PRGT.

Few reports have included producer-reported information on the on-farm interventions applied in outbreaks of PRGT. There are current recommendations (MLA 2007) but it is unclear if these recommendations are being implemented on a wide scale. Several farmers expressed a reluctance to undergo a program of extensive pasture renovation on their property. Reasons for this varied from concerns about pasture persistence, regrowth of WT pasture and suitability of paddocks for renovation. However all these concerns are probably best summarised by farmer scepticism about a clear cost-benefit ratio from pasture renovation. Finally one property had suffered a significant outbreak of PRGT from regrowth of wild type endophyte grasses in a renovated paddock.

Producer’s responses regarding their practices of animal management during PRGT outbreaks focused on several key areas which fell in line with the current recommendations: 1) where possible, sheep were moved to lower toxicity pasture, 2) farmers avoided stress to the animals where possible and frequently delayed or abandoned routine husbandry procedures during an outbreak; 3) some farmers were using licks containing commercially-available toxin binders in an attempt to reduce toxin absorption from toxic pasture. None of these strategies was reported to be 100% effective in reducing the prevalence of clinical cases on the properties visited. This was either due to a lack of availability of non-toxic pasture or because the disease was already
severe at time of detection. Reports on the efficacy of toxin binding licks or supplements were mixed. Licks were used typically in a situation where moving stock to alternative pasture was not a viable option. On all properties using licks signs of PRGT had continued and it was unclear if there had been any significant abatement of disease expression. Cost was also reported to be a significant issue for farmers implementing this strategy for large flocks over many calendar months.

Several farmers discussed the need to fence off or remove animals from paddocks with dams to reduce chances of death by drowning. Reports of large scale drowning during acute PRGT outbreaks gave rise to the recommendation to restrict dam access to livestock during these critical periods. However, although restriction of access to water in dams or streams is likely to be useful in reducing incidence of drowning in severely intoxicated animals it must be balanced with the need to provide animals with improved access to water particularly as results from this study and that of Sewell et al. (2011) suggests that animals consuming wild-type endophyte infested perennial ryegrass may have increased fluids requirements. A better understanding the behavioural changes associated with PRGT in livestock may assist to define appropriate fluid delivery for animals affected in severe outbreaks and so avoid the risk of stock losses by either drowning or dehydration. In Australia high ambient temperatures and low pasture moisture content combine with the need for animals to graze over a greater area to meet basic nutritional requirements may be key factors in increasing animal losses in severe outbreaks, rather than increased toxin exposure.

Conclusions
Although there is little doubt that novel endophyte varieties, lacking production of the toxins associated with PRGT, will play an important role in any future management strategies, these pasture types do have a number of limitations. Not all paddocks are suitable for pasture renovation; some novel endophyte varieties have poor persistence and/or pest resistance and may also have a toxicity spectrum that is unacceptable in certain situations. Therefore there is a need to consider other management options for producers affected by PRGT on an intermittent, but consistent, basis. Individual care of intoxicated animals is, from this study and producer reports, a successful strategy to improve survival in severely affected cases but has obvious limitations due to high labour costs. Toxin binders have potential to play a role in reducing subclinical toxin effects on production however observations of on-farm use of these products during the 2011 outbreak suggests that their efficacy in reducing clinical signs in moderate or severely affected cases is limited due to the requirement for the animal to ingest the binder independently when their normal feeding behaviours are severely impaired due to intoxication. Strategies to improve access to water and nutrients may play an important role in reducing the physiological stress of intoxication as well as assisting toxin clearance from the affected animals. However, survival
outcomes could also be assisted by pharmacological agents to reduce tremor and anxiety in moderate or severely affected animals. Barbiturates and benzodiazepines have been shown to reduce tremor by non-specific suppression of neurological activity, however they are short acting drugs in sheep and produce significant sedation which compounds management issues. Other pharmacological candidates need to be investigated which could provide a useful addition to the producer or veterinarian’s toolbox. Together these measures could assist producers to reduce animal losses during typical and atypical outbreaks.

Acknowledgements
The authors wish to acknowledge the significant contribution of all the involved in this study. We thank Shane Raidal and Leslie Weston for instructive comments on the manuscript. The authors also wish to acknowledge the excellent technical assistance of Craig Farish and staff of the CSU Veterinary Diagnostic Laboratory. MDAC is supported by a Research Higher Degree Assistance Scholarship from Meat and Livestock, Australia and the School of Animal and Veterinary Sciences, CSU. JQG is supported by a research fellowship from the EH Graham Centre for Agricultural Innovation.

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**Figure 1.** Sheep E1. This animal was ambulatory with Type I anaemia. Here it is restrained briefly for blood sampling after falling. Blood sample revealed moderate dehydration and anaemia due to access to ample green feed.
Chapter 10.7, Appendix 7: Papers, Conference Proceeding and Poster Presentations related to Thesis

Figure 2: Animal D10. This animal had been recumbent for several days at the time of examination. Note the profound muscle wasting.

Figure 3: Cerebellar Section from animal A1. Purkinje cell (P) with a prominent proximal axonal body or torpede (*). These torpedes are a feature of FRG T although they can occur with other forms of neurological disease (microphotograph at 100× magnification).
Table 1. Clinical findings from 13 sheep suffering from perennial rye grass toxicosis sampled during autumn 2011. Normal ranges are in (brackets). Values out with normal ranges are in bold. All animals exhibited clinical and histopathological signs consistent with PRGT. Those euthanized due to severity of their neurological signs are noted with #. Values for Animal 1 and 10 suggest renal failure. Abbreviations: Na, sodium; Cl, chloride; K, potassium; CK, creatine kinase; AST, aspartate aminotransferase

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10.7.6 Conference Proceedings: 9th International Symposium on Fungal Endophytes of Grasses and 1st International Symposium on Plant Microbiomes

VALIDATION OF A LARGE ANIMAL MODEL FOR INVESTIGATING PERENNIAL RYEGRASS TOXICOSES IN SHEEP

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Objectives

Perennial Rye Grass Toxicoosis (PRGT) is caused by livestock ingesting the complex mixture of toxins present in *Neotyphodium lolii* infested perennial rye grass. In Australia, PRGT causes significant morbidity and mortality in sheep during severe outbreaks and there is evidence that subclinical effects of this toxicosis have ongoing impacts on animal production, even in years where overt outbreaks are limited (Lane 2015). The key observable signs presenting in outbreaks of PRGT are neurological in origin (Cunningham 1959), caused by ingestion of the indole diterpenoid neurotoxin lolitrem B (Tor-Agbidye et al 2001) manifesting as behavioral changes, ataxia and tremor. Severe cases can result in an inability to stand or walk resulting in dehydration and death.

Currently no therapeutic treatment is available that can be delivered at a flock-wide level. Platforms for drug testing applicable to this complex neurotoxicity have been difficult to
establish in the target species due to seasonal variations in lolitrem B and ergovaline concentrations in pasture-based feeds (Reed et al 2011), and the high proportion of the vasoactive toxin ergovaline in PRG seed which results in ergovaline toxicity predominating (Johnstone et al 2012). This study will present data establishing a reproducible and reliable model of PRGT in sheep utilising a feed containing high levels lolitrem B toxin. This model excludes the confounding effects of high levels of ergovaline in previous models and establishes a reliable model for the naturally occurring disease in sheep.

Methods and Results

A controlled clinical trial was undertaken in which the clinical signs of PRGT were induced in male lambs by ingestion of a known dose of lolitrem B toxin only in feed. Animals were maintained on toxic feed for up to 35 days during which time movement, tremor and other physiological parameters, including serum biochemistry and urinalysis, were monitored to evaluate physiological responses to the toxin. Clinical signs were correlated with histological changes in the brain of affected animals at post mortem.

Animals receiving toxic feed showed comparable clinical signs to those presenting in naturally-occurring field cases (Combs et al 2014). These included mild tremor of the head and neck, significant tremor in the limbs which was exacerbated on intention, a heightened stress response and failure to make appropriate behavioral choices. Intoxicated animals showed a clear progression from stage 1 to stage 2 movement disorder (Combs et al 2014), finally proceeding to falling when pushed to move. Serum biochemistry identified mild changes in electrolyte balance, and clear histological lesions were observed in the cerebellum. Together these finding identified that a reliable model for PRGT had been established.

Impact

This trial identified a reproducible and reliable method for initiating the neurological signs associated with ingestion of lolitrem B in sheep. The naturally occurring disease was evident in these animals both at a behavioral, clinical and histological level. As outbreaks of perennial ryegrass toxicosis in ruminants cause significant financial losses for production industries (Bluett et al 2005, Lane 2015), establishment of this model provides a reliable platform on which to improve our understanding of the pathogenesis of this disease. Pharmaceutical products or feed additives that can improve clinical outcomes, reduce
mortality and improve productivity in both Australian and New Zealand production systems can now be tested using this experimental method.

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Control/Tracking Number: 2013-S-10803-SfN
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Current Date/Time: 5/8/2013 11:18:53 PM
The tremorgenic indole-diterpenoid toxin lolitrem B induces behavioural despair and activates stress and anxiety centres in the brain
Author Block: *J. C. Quinn¹, M. D. Combs², A. S. Hamlin³;

Abstract:

Lolitrem B is an indole-diterpenoid alkaloid toxin found primarily in perennial ryegrass. It is a potent inhibitor of large conductance Ca+ activated potassium (BK) channels. Ingestion of lolitrem B toxin causes cerebellar ataxia, tremor and Purkinje cell lesions in grazing livestock, a condition known as ‘perennial ryegrass staggers’. It has been suggested that a more widespread syndrome exists which includes anxiety, hyperaesthesia, allodynia and cognitive dysfunction in affected livestock. The aim of this study was to investigate behavioural changes associated with lolitrem B toxicoses using behavioural testing and c-Fos immunohistochemistry to determine cognitive and behavioural effects of lolitrem B toxicity and determine the level of activation in cells within neuronal stress pathways following acute lolitrem B exposure using the mouse as a model system.

Adult mice were injected with lolitrem B (2mg/kg, IP) or vehicle (DMSO). Tremor was analysed using a pressure sensor and ADI PowerLab ™ analyser. Spatial memory and
learning, object recognition, and motor function were tested using the AnyMaze™ system. Additionally, at three hours post injection four mice from each group were anaesthetised and transcardially perfused with 4% paraformaldehyde. Brains were sectioned at 40μm and c-Fos immunoreactivity was revealed using the avidin-biotin-horseradish peroxidase technique.

Analysis confirmed tremor in the range of 11-17Hz at one hour and increasing to 18-25Hz by three hours, peaking at nine hours post toxin exposure. A single dose of lolitrem B did not induce spatial learning or memory deficits despite appearing to induce short term abnormalities in normal exploratory behaviour. Significant behavioural despair was noted in all intoxicated animals such that they ceased all normal activities and remained stationary for extended period of time. Counts of c-Fos-IR nuclei revealed a significant increase in the nucleus tractus solitarius, ventromedial medulla, parabrachial nucleus, central amygdala, and paraventricular hypothalamus following acute lolitrem exposure. No c-Fos-IR was detected within the cerebellum.

Together, outcomes of these studies show that lolitrem B induces motor and behavioural changes consistent with previous clinical and experimental observations of lolitrem B intoxication induced anxiety behaviours in production livestock which is likely to result from activation of key stress pathways in the brain.
10.7.8 Conference Proceedings: Perennial Ryegrass Toxicosis; Investigating the Complex Biochemistry of Shaky Sheep. RACI, NPCG, 2019

Perennial ryegrass toxicosis; investigating the complex biochemistry of shaky sheep.

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Perennial Ryegrass Toxicosis (PRGT) is a common disease entity in Australian livestock systems. Clinical signs of PRGT include alterations in normal behaviour, ataxia (‘staggers’), ill thrift, electrolyte disturbances and gastrointestinal dysfunction (scours). The clinical signs associated with ingestion of toxic perennial rye grass are caused by the neurotoxin Lolitrem B, an indole–diterpenoid alkaloid produced by the endophytic fungus Neotyphodium lolii, a symbiont of perennial ryegrass. Perennial ryegrass is a pasture species with widespread prevalence across south-east Australia, Tasmania and New Zealand. Cases of PRGT can range in severity; animals can exhibit mild gait
abnormalities and a general failure to thrive, to those who present with severe movement abnormalities, seizures, lateral recumbency and death. Mild outbreaks of PRGT result mainly in subclinical production losses, management challenges and animal welfare issues for affected stock whilst severe outbreaks can result in significant stock losses. In either scenario there are significant economic losses for the affected producer.

Lolitrem B has a widespread activity in the central nervous system and is a potent inhibitor of large conductance Ca+ activated potassium (BK) channels, causing direct effects on neuroexcitation and neuronal function. To better understand the role of this toxin in the pathogenesis of PRGT we have examined its effects in both small and large animal models of the disease, showing that the clinical signs associated with intoxication can be accurately recapitulated in our model systems. To better define the systemic activity of Lolitrem B, we have also developed simplified and efficient methods for recovery of toxins from intoxicated animals, with up to 1000-fold greater sensitivity in detecting these analytes. Recovery of the compounds from mammalian tissue samples were found to be greatly facilitated using extraction by grinding in liquid nitrogen (Bligh-Dyer) with improved quantitation using reversed-phase HPLC coupled with mass-spectrometry (LC-MS). This could also be achieved using standard solvents (e.g. acetonitrile and water) and columns (e.g. C18), negating the use of halogenated solvents and columns previously described in the literature. This increased sensitivity has allowed identification of Lolitrem B toxin in the kidney, a previously unconfirmed target of the toxin. Together, these studies will allow a greater understanding of the systemic effects of lolitrem B in perennial rye grass toxicosis, as well as having potential applicability to other complex neurotoxicities affecting domestic livestock.
The tremorgenic indole-diterpenoid toxin lolitrem B activates stress and anxiety centres in the brain

Jane C. Quinn, Martin D. Combs, Adam S. Hamlin.

Introduction

Lolitrem B is an indole-diterpenoid alkaloid toxin found primarily in perennial ryegrass. It is a potent inhibitor of large conductance Ca2+ activated potassium (BK) channels. Injection of lolitrem B into the cingulate area of rat brain elicits a stereotypic Behavioural and Physiological responses known as BK channel agonist effects. It has been suggested that a more widespread syndrome exists which includes anxiety, hypothermia, atony and cognitive dysfunction in affected livestock. The aim of this study was to investigate Behavioural changes associated with lolitrem B exposure using survival testing and integrated home-cage behaviour to determine cognitive and Behavioural effects of lolitrem B toxicity and determine the level of activation in cells within neuronal stress pathways following acute lolitrem B exposure using the mouse as a model system.

Methods

8 adult mice were injected with lolitrem B (2mg/kg 4P) or vehicle (5% DMSO/5% PBS) (n=4). Tenor was monitored using a pressure sensor and AD1 Powerlab™ analyser. Tenor was analysed by selecting one minute epochs for Fast Fourier Transform (FFT) analysis. The rate of power output at high tenor frequencies (20-200Hz) to total power output (0-200Hz) was used as an estimate of tenor. Spatial memory and learning object recognition and motor function were tested using the Any-maze™ system. Additionally, at 3 hours post injection, 4 mice were killed and transcardially perfused with 4% paraformaldehyde. Brains were sections at 40µm and c-fos immunohistochemistry was revealed using the mouse-visual-horseradish-peroxidase technique (1:5000, Santa Cruz Biotechnologies, TX, USA).

Results

Analyses confirmed a tenor in the range of 10-100Hz. Lolitrem B induced a tenor of prolonged duration. Induction of tenor is also accompanied by a marked decrease in voluntary movement.

![Image](image_url)

Tenor analysis: FFT frequency analysis from a mouse prior to (A), and 3 hours post lolitrem B injection in kg (B). Tenor frequency (B) for total power output represents primarily voluntary movement. This is markedly decreased after injection (C) and 3 hours post (D). Tenor is increased in the intubated system where there is a distinct peak in a tenor band of 10Hz. (E) Tenorlolitrem B power also reveals tenor centre accounting for an increasing proportion of movement even at 1 hour. (F) Tenorlolitrem B heart rate at one hour post in s/peaked tested rats (P<0.05, green) & c-fos antibody (grey).

![Image](image_url)

Behavioural study Results

A single dose of lolitrem B did not induce spatial learning or memory deficits with respect to appearance time. Short-term and extended period memory was noted in all tests and animals such that they scored all normal activities and remained stationary for extended period of time.

![Image](image_url)

Memory task performance on demonstrating normal guia andserial taught (S) patterns in control animals compared to a more precise (C) normal pattern of serial taught (T) as demonstrated in olfactory and neural evolution. (D) Tenorlolitrem B injection increased the Hofilakina tenuis (H) and cross production (K) (P<0.05).

Conclusions

Together, these data shows that lolitrem B induces motor and behavioral changes consistent with previous clinical and experimental observations of lolitrem B intoxication induced anxiety主持召开 in two in post mortem brain which is likely to arise from activation of key stress pathways in the brain.

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Pharmacokinetics of bromide in adult sheep following oral and intravenous administration

TA Quest, MD Combs and SH Edwards

Objective To determine the pharmacokinetics of bromide in sheep after single intravenous (IV) and oral (PO) doses.

Procedure Sixteen Merino sheep were randomly assigned to two treatment groups and given 120 mg/kg bromide, as sodium bromide IV or potassium bromide PO. Serum bromide concentrations were determined by colorimetric spectrophotometry.

Results After IV administration the maximum concentration \(C_{\text{max}}\) was 82.21 ± 93.61 mg/L, volume of distribution \(V_{\text{s}}\) was 0.289 ± 0.031 L/kg and the clearance \(CL\) was 0.836 ± 0.055 mL/h/kg. After PO administration the \(C_{\text{max}}\) was 453.36 ± 43.27 mg/L, and the time of maximum concentration \(T_{\text{max}}\) was 108 ± 125 h. The terminal half-life \(T_{1/2}\) of bromide after IV and PO administration was 387.93 ± 115.35 h and 346.72 ± 94.05 h, respectively. The oral bioavailability \(F\) of bromide was 92%. No adverse reactions were noted in either treatment group during this study. The concentration versus time profiles exhibited secondary peaks, suggestive of gastrointestinal cyclic redistribution of the drug.

Conclusions and clinical relevance When administered PO, bromide in sheep has a long half-life \(T_{1/2}\) of approximately 14 days, with good bioavailability. Potassium bromide is a readily available, affordable salt with a long history of medical use as an antiasthmatic, sedative and antiseizure therapy in other species. There is a number of husbandry activities and flock level neurological conditions, including perennial ryegrass toxicosis, in which bromide may have therapeutic or prophylactic application.

Keywords bromide; pharmacokinetics; sheep

Abbreviations AUC\(\text{IV}^{\text{IV}}\) area under the curve; AUMC\(\text{IV}^{\text{IV}}\) area under the first moment curve; \(C_{\text{max}}\) maximum concentration; \(CL\) terminal elimination rate constant; CEC, extracellular fluid; LD, loading dose; PK, pharmacokinetics; PD, pharmacodynamics; PET, phantom half-life; \(T_{1/2}\) time to maximum concentration; \(V_{\text{s}}\) initial volume of distribution; \(V_{\text{t}}\) terminal volume of distribution.

In veterinary medicine, potassium bromide (KBr) has found renewed favor in recent years as a therapeutic agent. In Australia, KBr is registered in dogs for the treatment of idiopathic epilepsy, and it is being increasingly used worldwide for seizure therapy as it is associated with fewer side effects than phenobarbital.\(^{28}\) It is also used extensively off-label by horse owners as a calming agent, sometimes in cases of compromised health, to date little research has been undertaken as to its efficacy and appropriate application.\(^{29}\)

The potential uses of Br in sheep have not been previously investigated. Because of its tranquilizing and anxiolytic actions, Br has the potential to address animal welfare and production issues associated with a range of husbandry procedures such as road and sea transportation, anxiety associated with shearing and antimicrobial associated with stress and introduction of new feeds.\(^{28,29}\)

Bromide also has a potential use as a therapeutic agent for neurological disorders that affect sheep at the herd level. For example, the potential of Br as a natural bufotenin, \(\beta\)-lactam B, was recently identified as a calcium-activated potassium channels antagonist, depolarizing neuronal membrane and thereby increasing neuronal excitability.\(^{28,29}\) Bromide’s action via chloride channels results in membrane hyperpolarization and Br also appears to potentiate GABAergic inhibition.\(^{28,29}\) These results suggest Br could be used prophylactically to reduce the incidence of perennial ryegrass toxicosis (PRGT) associated with \(\beta\)-lactam B.

The distribution of Br in sheep has been partially elucidated. IV \(\beta\)-Br was used to estimate extracellular fluid (ECF) volume and was found to exhibit delayed distribution kinetics.\(^{28,29}\) Essentially, Br is initially confined within the vascular space, with extracellular movement into interstitial fluid being slow, taking 2-3 h to equilibrate with the ECF. Br equilibration with serum fluid is even slower, with only a 4-9% entering the non-dialyzable fluid and taking 24 h for full equilibration to be reached.

In the course of drug development, both pharmacokinetic (PK) and pharmacodynamic (PD) studies are conducted in order to guide design of optimal dosage schedule and administration interval for subsequent clinical trials. From PK studies in a small number of animals, using a test dose (not necessarily a predicted therapeutic dose), the concentration versus time profile is generated. From these simple plots, estimates of the necessary PK parameters may be derived, which are subsequently used to determine a dose regimen. Target drug concentrations (within the therapeutic range) are determined from PK studies, involving dose-response experiments (in vitro and in vivo dose titration). The use of such a PK-PD approach offers rational guidance in attempting to streamline the drug development process, with the aim of reducing animal welfare stemming from large scale dose titration studies.\(^{28,29}\) Moreover, once the
PK parameters are known, it is a simple matter to use PK/PD integration to generate a patient dose regimen for multiple applications of a particular drug. Should a therapeutic have a novel application potential, the new target concentration is applied to the existing PKs.

Of prime importance in this study was to determine the PK parameters of each clinical treatment. A relatively long terminal elimination half-life (t1/2) is important for an extended duration of action; activity is also used to estimate withholding periods, vitally important in any drug used as a food additive. The interval used is the duration between dosing. A dose is calculated using the formula for the dosing target concentration (tD) is the parameter used to calculate the dosing dose (ID) to each target drug concentration tD quickly. For the first time, this study investigated the pharmacokinetics of hir in sheep, as the basis for future studies of its therapeutic application.

Materials and methods

Animals
Sixteen 6-year-old merino ewes (weights 49.5–67 kg; average body condition score 2) were allocated equally to 40 (treatment group) and 30 (control group) plus two coteries of two sheep. Of the sexed ewes, all sheep were mated in a pen then put through a race, with every second sheep assigned to pen B. All sheep were weaned at 6 weeks of age and 6 weeks was the same time. All sheep were housed in individual feeding pens and fed twice daily on a ration of oats and lupins, with ad libitum hay and water. Estimated chloride content of the oats and lupins was 0.45% and 0.48%, respectively.

A 105-week, 32-cm IV cannula (Perena, Certo Scheda 335, Muhlingen, Germany) was placed in the left jugular vein and secured with a 25G polypropylene suture. A 25 cm IV tube (IV set) was connected to the cannula hub and the tube was then bonded.

Sodium bromide (NaBr Sigma-Aldrich, St. Louis, MO, USA) and potassium bromide (KBr Sigma-Aldrich) solutions were prepared using sterile water. The prepared NaBr solution was then filtered through a cellulose (0.22 µm, MILLESFIL, USA) filter. Sodium and potassium bromides were administered to each sheep with 120 mg/kg of NaBr (134.6 mg NaBr and 178.8 mg KBr). All serum concentration of Br and KBr was determined directly from the samples. Other PK parameters were determined for each sheep by non-compartmental analysis using a commercial software program (Tilgate Ltd, Quanta Fischer Verlag). Arterial blood samples were taken at each time point for serum sodium and potassium analyses. Arterial blood samples were taken at each time point for serum sodium and potassium analyses.

Administration route

Ivrousceous. The NaBr solution was administered through the cephalic vein using a 21G needle over a period of 1 min. Sheep were restrained in a seated position. Blood samples were collected at 0, 1, 3, 6, 8, 10, 12 and 24 h. Samples thereafter were collected at 12 h intervals up to 24 h and then at 24 h intervals up to 336 h. A total sample was taken at 336 h. For each sample, the 5 ml of blood collected was used to enrich the electrode used to introduce 1 ml of blood, which was then placed into a glass screw-capped tube (Vacutest, Greiner Bio-one, Kremsmünster, Austria). The samples were stored at 4°C for about 30 min before centrifugation at 2000g for 5 min. Serum was harvested and stored at −20°C until analysis.

Oral. The KBr solution was administered via an orogastric tube, which was flushed with 500 ml of water. Blood samples were collected at 0, 1, 3, 4, 6, 8, 10, 12 and 24 h. Then at 12 h intervals up to 24 h and then at 24 h intervals up to 336 h. A total sample was taken at 336 h. When collecting blood samples at 1 h through to 10 h the ram was allowed to have the blood collected as the oral dose, but if the blood load had affected the animal mortality.

Determination of serum bromide concentrations

Serum Br concentrations were determined by colorimetric spectrophotometry as previously described, with some modifications. Briefly, 0.35 ml of serum was added to 3.15 ml of 10% metaphosphoric acid (Sigma-Aldrich) in a 10 ml centrifuge tube, vortexed, then centrifuged for 15 min at 2000g. Next, 2.5 ml of supernatant was added with 0.35 ml of 0.5% Anf. B, (Sigma-Aldrich) and left to stand for 30 min. Absorbance was measured with a spectrophotometer at 460 nm. The standard curve was linear in the range of 25–3690 µg/ml, R² = 0.9992. The lower limit of quantification was 25 µg/ml.

Pharmacokinetics

Mean concentration-time (Cmax) of Br and time to Cmax (Tmax) were determined directly from the data. Other PK parameters were determined for each sheep by non-compartmental analysis using a commercial software program (Tilgate Ltd, Quanta Fischer Verlag). Arterial blood samples were taken at each time point for serum sodium and potassium analyses. Arterial blood samples were taken at each time point for serum sodium and potassium analyses.

Clarence (Cl) was calculated using the equation:

\[
\text{Cl} = \frac{C_{\text{Dose}}}{C_{\text{initial}}} 
\]

Mean residence time (MRT) was calculated using the equation:

\[
\text{MRT} = \frac{AUMC}{AUC_{\text{last}}} 
\]

The terminal volume of distribution (Vₜ) was calculated using the equation:

\[
Vₜ = \frac{C_{\text{Dose}}}{\text{Cl}} 
\]

The terminal volume of distribution (Vₜ) at pseudo-equilibrium was calculated using the equation:

\[
Vₜ = \frac{C_{\text{Dose}}}{\text{Cl}} 
\]

The t₁/2 was calculated using the equation:

\[
\text{t₁/2} = \frac{\ln 2}{\text{Cl}} 
\]

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Chapter 10.7, Appendix 7: Papers, Conference Proceeding and Poster Presentations related to Thesis

**PRODUCTION ANIMALS**

Whereas parameters $C_{max}$, $T_{max}$, and AUC (where bioavailability is not absolute) are expected to differ when given IV or PO, $\phi$ should be the same, regardless of the route of administration. A T-test of the hypothesis of no difference between the $\phi_i$ population means was performed. All results are expressed as mean ± standard deviation.

**Results**

**Animals**

No discernible neurological effects were seen in sheep in the PO group. No abnormal signs of neuromotor or dysmotility were recorded and animals continued to eat and drink. Assessment of any acute neurological effects correlating with peak Br concentration following IV administration was difficult because the sheep were held in the recumbent position throughout the initial 20 min for ease of sampling. Following IV injection, all sheep walked back to their individual pens and observers subjectively reported a mild tranquilizing effect for approximately 1–2 h post-injection.

**Pharmacokinetics**

The relevant non-compartmental pharmacokinetic parameters derived from this study are summarised in Table 1. The concentration–time profiles for IV and PO serum Br are shown in Figures 1 and 2, respectively.

The $t_{1/2}$ of Br in sheep following PO administration was 14.4 days and the IV $t_{1/2}$ was 16.2 days; however, the difference between groups was not statistically significant ($T = 0.73, d = 14, F = 0.095$).

**Table 1. Pharmacokinetic parameters (mean ± SD) of bromide after intravenous and oral administration to eight sheep at a dose of 120 mg/kg**

<table>
<thead>
<tr>
<th>Pharmacokinetic variable</th>
<th>Intravenous administration (mean ± SD)</th>
<th>Oral administration (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{max}$ (mg/L)</td>
<td>822.11 ± 95.61</td>
<td>453.86 ± 43.37</td>
</tr>
<tr>
<td>$T_{max}$ (h)</td>
<td>33.72 ± 5.32</td>
<td>10.8 ± 124.66</td>
</tr>
<tr>
<td>AUC, ($\mu$g/ml/h)</td>
<td>157272.6 ± 52681.53</td>
<td>164168.0 ± 241756.16</td>
</tr>
<tr>
<td>$MRT_{total}$ (h)</td>
<td>545.5 ± 226.1</td>
<td>41.3 ± 150</td>
</tr>
<tr>
<td>$D$ (mL/kg)</td>
<td>0.836 ± 0.255</td>
<td>--</td>
</tr>
<tr>
<td>$V_1$ (L/kg)</td>
<td>0.286 ± 0.031</td>
<td>--</td>
</tr>
<tr>
<td>$V_2$ (L/kg)</td>
<td>0.399 ± 0.102</td>
<td>0.899 ± 0.017</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>387.09 ± 115.35</td>
<td>346.72 ± 94.05</td>
</tr>
<tr>
<td>$F$</td>
<td>--</td>
<td>0.92</td>
</tr>
</tbody>
</table>

$C_{max}$, area under the curve; $D$, clearance; $C_{max}$, maximum concentration; $F$, oral bioavailability; $MRT_{total}$, mean residence time; $t_{1/2}$, terminal elimination half-life; SD, standard deviations; $T_{max}$, time of maximum concentration; $V_1$, initial volume of distribution; $V_2$, terminal volume of distribution at pseudo-equilibrium.

**Figure 1.** Serum concentration (mean ± SD) of bromide after intravenous administration to eight sheep at a dose of 120 mg/kg.
Chapter 10.7, Appendix 7: Papers, Conference Proceeding and Poster Presentations related to Thesis

These were numerous pronounced peaks in the PO concentration versus time curve following the initial absorption phase. These peaks approached the Cmin seen on day 1, but occurred many days later (Figure 2). Indeed, some sheep had Cmin values well beyond the initial PO absorption phase. Similar but smaller peaks were also seen with IV Br (Figure 1).

Bromide was absorbed rapidly following PO administration. Prior to this study, it was predicted that absorption might be delayed because of dilution, the rumen having an estimated volume of 10-12% kg of body weight. However, the Cmin in this study was similar to that measured in horses given a comparable oral dose of Br.7 Ruminal epithelial cells express large conductance chloride channels permeable to chloride8 and the rumen has a high chloride absorptive capacity, even against the net electrochemical gradient for the ion.9,10 It is likely that these high conductance channels are also responsible for overcoming a relatively lower initial Br concentration in the ruminal gastric fluid compared with monogastric species.

The life of Br in sheep, of approximately 14 days, is comparable with the 12 days in horses11 and the 12-24 days in the dog,12 but is considerably longer than the approximately 3-days in the horse.7 This long elimination phase is a function of slow excretion. Br is not metabolised and is subject to extensive renal tubular reabsorption, the net result being the anion is continually recycled throughout the body.13

In the classic PO pharmacokinetic profiles of monogastric species, secondary peaks are uncommon and are usually a function of unusual drug metabolism, in particular enterohepatic recycling. Bromide, however, is not metabolised, so possible causative mechanisms are limited to absorption, distribution and elimination. A study conducted in horses, using a similar PO dose of Br to that used in this study, produced a profile without secondary peaks in serum concentration.5

The secondary peaks phenomenon is possibly related to ongoing rumen and omasum reabsorption, a function of bidirectional Br flux through chloride channels. Bromide has been shown in previous studies to move into ruminal fluid subsequent to IV administration.13

Similar secondary peaks were also seen following IV administration, albeit much less pronounced (Figure 1). However, it is notable that the IV peak appeared to occur at similar time points to those seen following PO administration.

Redistribution becomes even more complex when ruminal salins volumes and the role of chloride in salins production are taken into account. Ruminant salins production is significantly higher than in most other species because it has an important role in providing fluid and buffer to the rumen. Salins production in the sheep is estimated to vary between 3 and 10 L/day depending on feed type.14,15 One study estimated that 190 ml. (1.4 L) of salins is typically produced when eating dry forage, such as that consumed in this trial.7 This is approximately twice the estimated plasma volume for sheep.

Transpyloric chloride movement is a driver of salins production; however, the predominant chloride channels in solitary mucin cells (i.e. Br secretion over chloride with Br secreted in salins at high concentrations).16 Given the large, but fluctuating volume of salins production and the selective uptake of Br by salins glands, it is possible that the fluctuations in serum Br concentration simply reflect the variation in salins production at the time of sampling.

The generalisation that Br is transported in the same way as chloride is useful, but probably overly simplistic. Just as Br is different electrochemically from chloride, there is also wide variation in its selectivity, affinity/sensitivity and tissue distribution among monogastric channels.16

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Further studies of the movement of Br in the rumen and saliva would be useful in better defining this process.

Secondary peaks for other drugs have been identified in ruminants. Artificial peaks in plasma concentrations were seen in 8 and 13 h after PO administration in sheep.13 The authors attributed these peaks to variable rates of absorption at multiple sites.13 However, gastrointestinal recirculation creating secondary peaks has been identified in monogastric species, including for nonsteroids in humans.14 A series of peaks was also seen following PO tranquil in sheep, with those authors suggesting variable absorption, or delayed absorption from the drug binding to particulate matter, as the cause.15

The mean bioavailability of Br in this trial was 92%, which is much higher than the estimated bioavailability of 32-36% in the horse8 and 40% in the dog.10 This greater bioavailability in the sheep is probably related to the prolonged rumen residence time of ingested feeds.16

The Vd of 0.286 ± 0.011 reflects the ICF space (although Vd data are not primary measures of physiological compartments, they do offer insight) and approximates the Vd of 0.365 L/kg used as an estimate of ICF in the sheep.17 The calculated terminal volume of distribution (Vd) when equilibration with rumen fluid is assumed, was 0.395 ± 0.012 and 0.386 ± 0.027 L/kg for IV and PO administration, respectively. That these measures are similar is unsurprising because Vd is a proportionality factor related to concentrations during the log linear phase of drug elimination, from which t1/2 is derived.

Volume of distribution is the parameter used to calculate the LD using the equation $LD = Vd \times C_0$, where $C_0$ is concentration steady-state, or the effective concentration for a particular use (as determined by PO studies). The Vd value is most appropriate when Br is to be given as a PO bolus, and Vd, in circumstances where it is to be given over a few days.

Because of the long t1/2 following PO administration, the serum Br concentration as a narrow range (0-300-600 mg/L) for 14 days. This long t1/2, with a two-fold difference between peak and trough values over a 2-week period confers great potential for therapeutic application, particularly prophylactic use.

The study dose of Br (120 mg/kg) resulted in sustained Br concentrations approximating one-third of the lower boundary of the anticonvulsant range (1.0-2.0 mg/L).18 Concentrations of Br that will prevent, attenuate or destroy PRT are unknown, although it would be assumed that in all but the most sensitive cases to be less than those required to protect against grand mal seizures. For PRT and other neurological conditions causing tremor or seizures, it may be necessary to provide Br in a larger LD than was used in this study. The use of large oral LDs has been a problem in monogastric species and although it is anticipated that the large capacity of the rumen will mitigate these concerns, further research in this area is required before the extent of application of Br can be determined. However, even with the relatively large, acute PO dose given in this study there appeared to be no side effects and all animals continued eating after oral dosing.

Concentrations of Br that will reduce anxiety and ameliorate the stress of transport are also unknown, but could notionally be assumed to be lower than those required to miss the acute threshold. Some anxiety

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**References**
