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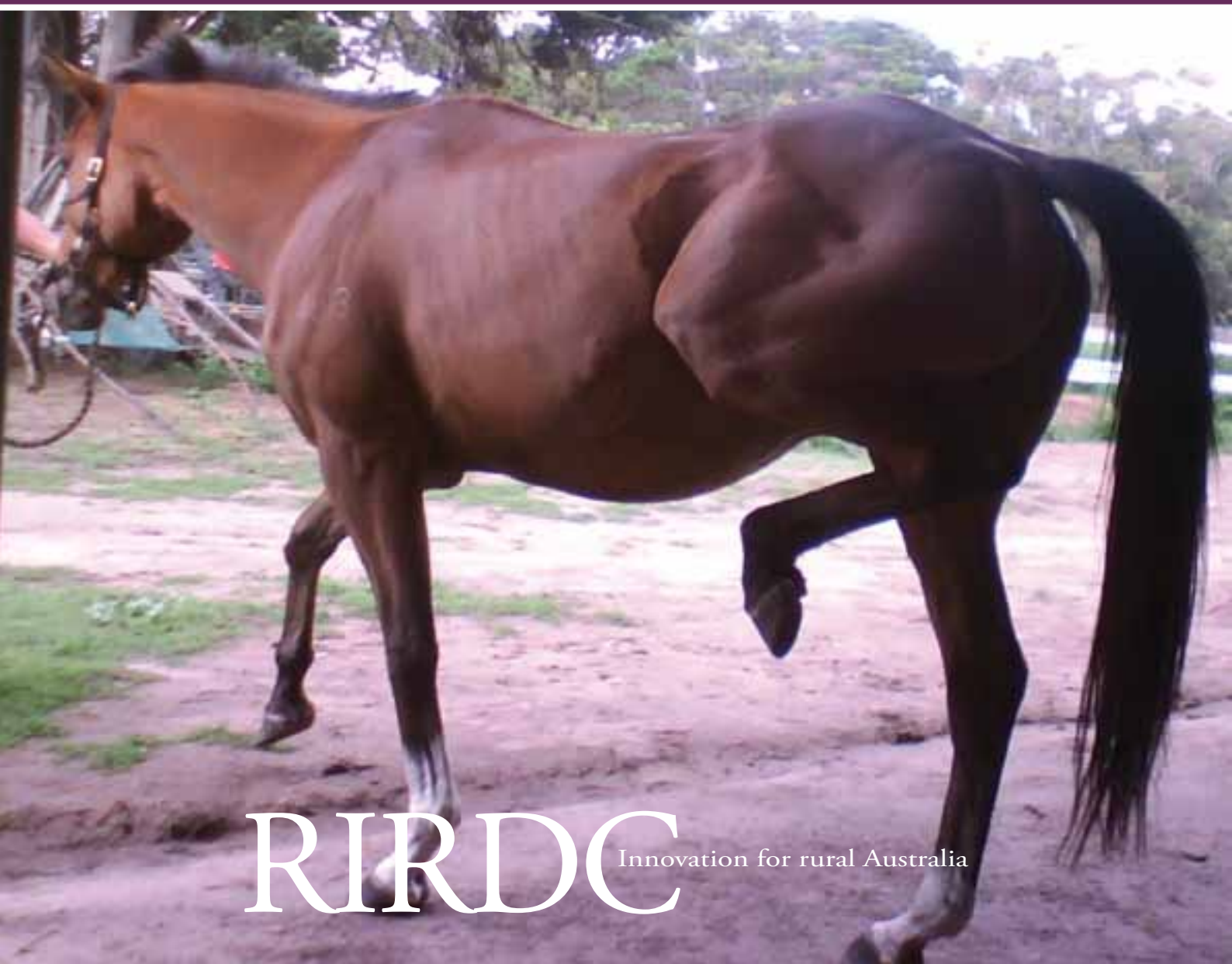
Investigation into the Cause of Australian Stringhalt

RIRDC Publication No. 11/127



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Investigation into the Cause of Australian Stringhalt

by Charles El-Hage

December 2011

RIRDC Publication No 11/127
RIRDC Project No PRJ-003742

ISBN 978-1-74254-306-2
ISSN 1440-6845

Investigation into the aetiology of Australian stringhalt
Publication No. 11/127
Project No. PRJ-003742

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Electronically published by RIRDC in December 2011
Print-on-demand by Union Offset Printing, Canberra at www.rirdc.gov.au
or phone 1300 634 313

Foreword

Australian Stringhalt (AS) is a debilitating condition of pasture based horses resulting in an abnormal gait and involuntary hyper (over) flexion of the hindlimbs when attempting to walk. It occurs in individual horses or in outbreaks. Prolonged dry summers on poorer quality soil types with unimproved pasture are recognised risk factors. The condition has been documented for over 120 years in south-eastern Australia and has been documented in several continents with little progress in determining the exact cause.

There are several common factors that are well reported in outbreaks of AS. They include a strong association with the presence of the flatweed *Hypochaeris radicata*. Most cases occur in late summer/autumn following prolonged dry spells with affected paddocks being typically of poorer unfertilised soil types. Although a strong association between the disease and *H. radicata* is well recognised there has been little published work to investigate potential toxins within or associated with the weed.

Extensive testing was carried out in this study to identify possible fungal toxins in flatweed associated with outbreaks of AS. No evidence of such mycotoxic alkaloids was found. This finding suggests that such alkaloids are unlikely to be responsible for the nerve damage observed in horses suffering from AS.

While further work to identify a specific toxin continues, horse owners could take steps to improve pastures to reduce the prevalence of *H. radicata* within paddocks where it is identified as a predominant species.

Funding for this project was made available from the RIRDC and the University of Melbourne's Faculty of Veterinary Science, Department of Land and Food Resources and Metabonomics Australia, School of Botany.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our Horse R&D program, which aims to assist in developing the Australian horse industry and enhancing its export potential.

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Craig Burns
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About the Author

Charles El-Hage is a researcher and lecturer in the Department of Veterinary Science at the University of Melbourne. Victoria Australia. He has co-authored several articles relating to intoxication of horses and livestock including:

- C El-Hage, MJ Lancaster. (2004). Mycotoxic nervous disease in cattle fed sprouted barley contaminated with Aspergillus clavatus. Australian Veterinary Journal, 82(10): 639-641
- CM El-Hage, LE Bradbury AF Dredge and J.McGregor (2010). A fatal outbreak of suspected botulism in twelve horses. Australian Equine Veterinarian. 29(1) Autumn 2010:50-54

Acknowledgments

This project was made possible largely due to the support of Professor Ron Slocombe. The support and assistance of Dr.Daniel Dias, Terra Stark, Nirupama Jayasinghe at Metabolomics Australia (School of Botany), The University of Melbourne. Dr Simone Rochfort DPI Victoria Agribiosciences, La Trobe University, Larry Walker SSPL Hamilton and Angus Tye of the Faculty of Land and Food Resources, University of Melbourne was invaluable.

Abbreviations

AS	Australian Stringhalt
BSTFA	*N*,*O*-bis(trimethylsilyl)trifluoroacetamide)
GC	Gas Chromatography
H. radicata	*Hypochaeris radicata*
HPLC	High Performance Liquid Chromatography
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
RH	Relative Humidity
SPE	Solid Phase Extraction
TIC	Total Ion Chromatogram
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl

Contents

Tables

Figures

Executive Summary

What the report is about

Australian Stringhalt (AS) is a debilitating condition of pasture based horses characterised by involuntary hyper (over) flexion of the hindlimbs when attempting to move. Although the condition occurs worldwide it has been particularly well documented for over a century in south-eastern Australia. In fact the pasture based or outbreak form of stringhalt is generally referred to as Australian Stringhalt.

Background

Previous workers have suggested that due to the sporadic occurrence of AS and its association with climatic conditions that a (fungal) mycotoxin associated with *Hypochoeris radicata* may be responsible. A number of mycotoxins have been well documented as causes of neurological disease in animals grazing affected pastures after prolonged dry weather conditions.

Aims/objectives

- To identify the presence or absence of toxic agent(s) responsible for Australian Stringhalt that may be present in, or associated with the weed *H. radicata.*
- Toxins within the plant possibly induced by environmental stress will be analysed, in addition to any mycotoxins (fungal poisons) associated with the plant.

Methods used

A series of chemical analyses to determine metabolite differences in *H. radicata* under varying environmental and growth conditions were performed. Comparisons of metabolite levels between plants were carried out using Gas Chromatography – Mass Spectrometry (GC-MS) analytical methods. Juvenile, well watered plants were compared to those from paddocks with horses suffering from AS following typical drought periods. Simulated drought stress was also applied to *H. radicata* under controlled growth chamber conditions for subsequent analysis.

Results/key findings

Analysis of chemicals within *H. radicata* identified many known and unknown compounds in young plants and older drought stressed ones.

The suggested role of (fungal) mycotoxins appears unlikely as a causal factor in AS despite several suggestions they were a possible cause of the condition. Analysis of *H. radicata* from two properties with AS revealed no evidence of alkaloids. The analysis was wide ranging encompassing many groups of compounds recognised as toxins associated with neurological disorders in grazing livestock.

The absence of mycotoxins would limit the usefulness as agents such as mycotoxin binders being administered to protect against the onset of AS.

Implications for relevant stakeholders for:

This study was a pilot one to assist in directing the areas for research into the possible role of *H. radicata* in AS. Preliminary findings have been encouraging in relation to elevation of several metabolites some of which are known toxins in drought stressed plants.

The isolation of unidentified primary and secondary metabolites possibly produced in response to drought stress confirms the epidemiological evidence that the flatweed *H. radicata* should be avoided by owners to assist the prevention of AS in grazing horses.

Until a particular toxin is identified horse owners and carers would be advised to reduce exposure to *H. radicata* in paddocks grazed by horses particularly after prolonged dry spells. The suggested role of (fungal) mycotoxins as a cause of Australian Stringhalt is unlikely despite several suggestions these were a possible cause of the condition. The practice of feeding mycotoxin binders to treat horses affected with AS or as a preventative measure is therefore unlikely to be protective.

Until a particular toxin is identified horse owners and carers would be advised to reduce exposure to the flatweed or false dandelion *H. radicata* in paddocks grazed by horses particularly during prolonged dry summers.

Recommendations

Over one million horses are estimated to be present in Australia the majority of which reside in paddocks in south-eastern Australia. Australian Stringhalt occurs in this environment particularly after long dry summers. Such weather patterns have been recorded as becoming more frequent, increasing the significance of such research. Horse owners and the various industries they participate in will be relieved of significant welfare, economic and performance loss issues if the cause of Australian Stringhalt is revealed.

Further more sophisticated analysis could be performed to identify these metabolites. It is anticipated that the potential for toxicity for many of these will be determined by exposure to horse nerve cell cultures.

Introduction

Australian Stringhalt is a condition of horses resulting in extreme hindlimb hyperflexion such that the limb may contact the belly when trying to move forward. The abnormal gait is a result of involuntary control of the hindlimbs and has caused great intrigue over several centuries (Kendall 1884; Pemberton and Caple 1980; Huntington, Jeffcott et al. 1989).

There has been a clear association identified between horses developing an extremely odd goose stepping like hindlimb gait and pasture weeds. The condition to this day still puzzles veterinarians and horse owners alike. Not only has the specific cause remained elusive, but the exact reason for such a spastic type gait is unclear.

Although studies have identified a nerve condition due to a distal axonopathy occurring in the longer nerves of the horse, the causative agent has not been clearly identified.

The conditions that are typical of most outbreaks of Australian Stringhalt have been well described and lead to the widely held belief the disease is caused by ingestion of a toxin. Most affected horses have been found to be grazing paddocks of generally poorer quality soils at the end of drier summers (Pemberton and Caple 1980; Cahill, Goulden et al. 1985; Huntington, Jeffcott et al. 1989).

Figure 1 *Hypochaeris radicata* demonstrating yellow flowers, branched tall stems and rosette leaf base.

Hypochaeris radicata is a yellow flowered perennial herb originally from Europe is a member of the Asteraceae family that now appears world-wide. A large often branched tap root enables survival in drier climates and often provides a competitive advantage in non improved pastures (Offord 2006).

Most studies implicate *Hypochaeris radicata* (commonly referred to as Flatweed) as being prevalent in paddocks where horses develop AS and often as the predominant weed (Cahill, Goulden et al. 1985; Huntington, Jeffcott et al. 1989; Araujo, Curcio et al. 2008; Domange, Casteignau et al. 2010). Removal of horses from these pastures results in gradual improvement over 6-8 months in the majority of cases (Pemberton and Caple 1980; Huntington, Jeffcott et al. 1989).

A recent study reported that, 65 of 66 six paddocks containing horses succumbing to AS contained *H. radicata*. This is the greatest numerical association of *H. radicata* to date and occurred following a prolonged drought in France (Domange, Casteignau et al. 2010). The same study reported that approximately 30% of exposed horses developed signs.

Despite this association previous studies have not been to reproduce AS until this decade (Seddon and Belschner 1926; Pemberton and Caple 1980; Araujo, Curcio et al. 2008).

Whether the plant is solely responsible for the presumed intoxication or conditions and factors associated with its presence is still under investigation. The association with prolonged dry periods has led some authors to suggest a possible mycotoxic (fungal) contamination of the flatweed (Pemberton and Caple 1980; Cahill, Goulden et al. 1985; Huntington, Jeffcott et al. 1989; Huntington, Seneque et al. 1991) as occurs with other mycotoxicosis in pastures such as ryegrass staggers.

Several workers have noted that certain stresses to the plant result in production of various toxins (Maruta, Fukushi et al. 1995) and that a secondary metabolite that horses are susceptible to, may be responsible for the nerve damage seen in AS (Domange, Casteignau et al. 2010).

Although the presence of *H. radicata* seems necessary for AS to occur, other factors are likely to be important as its presence in a pasture alone does not appear to be sufficient to cause AS. Other factors may be environmental or a combination of these and/or susceptibility to damage by certain host factors/deficiencies.

These observations have lead to great interest in the possible toxicity that may develop in, or is associated with *H. radicata*. Our study aims to answer at least some of these long-standing questions.

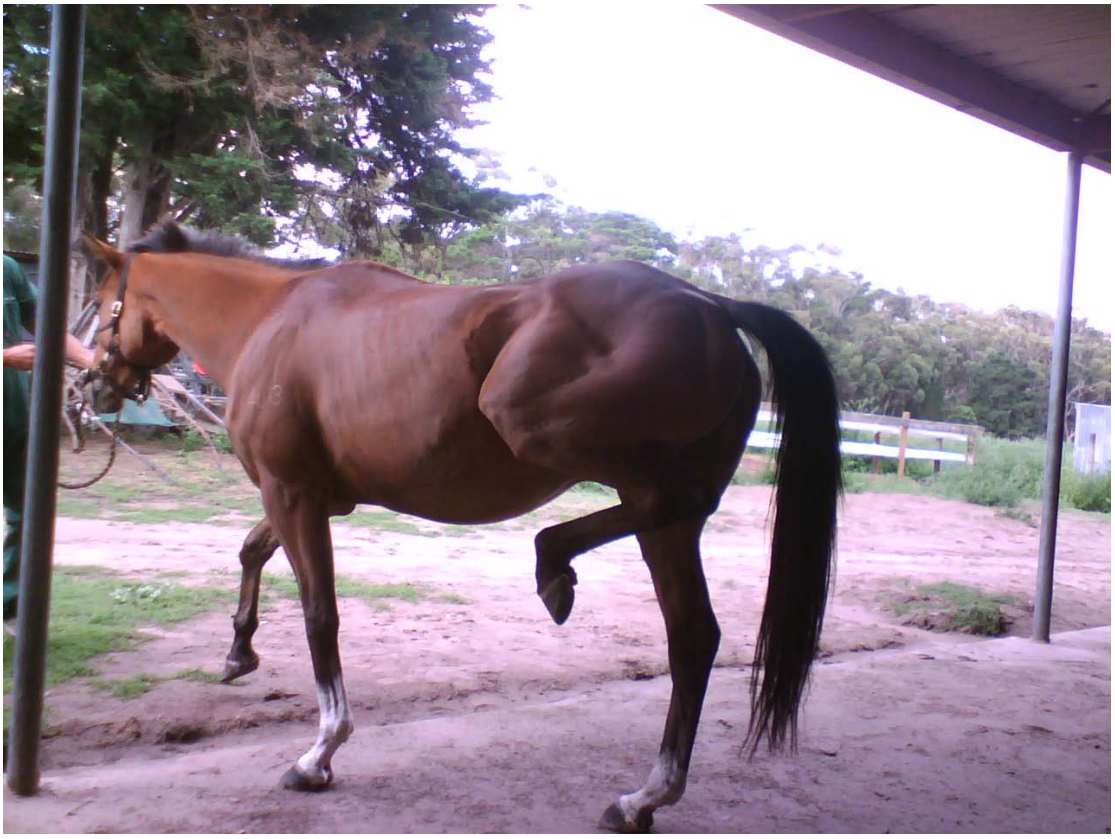

Figure 2 Horse suffering from Australian Stringhalt demonstrating prolonged hyperflexion of left hind limb. This was one of three horses grazing plants from sample 1B.

Objectives

- To establish whether *H. radicata* is a potential source of neurotoxins a thorough analysis of the plant from a range of environmental conditions and growth stages was conducted to maximise detection of potential toxins.

- By testing plants from a variety of environments and growth stages, differences in analytes between test and control plants were examined.

The association of outbreaks of AS with prolonged dry summers may suggest changes within and or associated with *H. radicata* following climatic stress. Collecting field samples from paddocks of horses suffering from AS following drought periods provides a positive control. These test samples will be compared to juvenile plants collected during favourable conditions (negative controls).

Furthermore, analysis of two batches of *H. radicata* that have been incubated under contrasting controlled conditions will be performed. Plants that have been well watered under simulated day/night summer conditions will be contrasted with those that have had restricted watering to simulate drought stress.

It is anticipated that changes may be detected as the plants undergo climatic stress if *H. radicata* produces toxic chemicals in response to drought conditions.

Detection of possible mycotoxins (fungal toxins) associated with field samples of *H. radicata* is to be carried out since the climatic conditions and sporadic nature of outbreaks of AS are suggestive of such toxins.

Methodology

Sample Collection - *Hypochoeris radicata*

Sampling of *H. radicata* was conducted at various field sites as follows:

Test Batches

Batch 1B: Bellarine Peninsula

- Sandy, poor quality unfertilised soil, little pasture mostly weeds and some Kikuyu grass. Horse 1 TBX (Buddy) seen to graze *H. radicata* developed signs in February after introduction to paddock in November. Two other unaffected horses were in the same paddock in poorer body condition. Harvested February 2010.

Batch 2B: Anglesea region

- Unimproved pasture recently fertilised, native and introduced grasses. Densely populated with *H. radicata.* Two Thorougbred horses developed AS approx 1 month after introduction to this pasture (see Figure 3 Below). Harvested March 2010.

Figure 3 Mature *H. radicata* from paddock with two horses that developed Australian Stringhalt.

Control Batches

Batch C: Healthy plants well watered flowering stage

- Harvested February Bellarine Peninsula 2010. Horses grazing no clinical abnormalities.

Batch JF: Healthy plants well watered preflowering

- Juvenile harvested February Bellarine Peninsula 2010.

Batch 4B: Neighbouring paddock to 2B (Anglesea)

- four to six horses had grazed this paddock and remained unaffected.
- Harvested March 2010.

Batch 5: Mature plants in paddock with horses grazing

- paddock with no obvious abnormalities. Harvested March (Werribee, VIC) 2010.

Batch 6: Healthy well watered flowering specimens.

- Harvested Bellarine Peninsula in March 2010.

Other control plants submitted were healthy well grown specimens

Mycotoxin screening

Six batches of *H. radicata* were collected for analysis 30/11/10. Samples marked as two test batches from AS affected properties and four various control batches. The first assay run was for Lolitrem B and similar, where a methylene dichloride extract was purified over a silica Solid Phase Extraction (SPE) and analysed by normal phase High Performance Liquid Chromatography (HPLC) with fluorescence detection (ex 265nm, em 440nm). The second assay run was for ergot-type alkaloids. An acidic extract was purified using an ion-exchange/C18 combination SPE and analysed by reverse-phase HPLC with both diode-array Ultraviolet detection and fluorescence detection (ex 310nm em 415nm).

The final assay run was a plant alkaloid screening method previously described (JR Frelich and Marten 1973). A chloroform/ammonia extract of the sample was prepared and back extracted into 1N sulphuric acid. An aliquot of this was then combined with silicotungstic acid to precipitate any alkaloids present and the resulting turbidity was approximately scaled by eye to give an indicative quantity.

Growth Chamber Experiment

Twenty juvenile *H. radicata* plants were placed in a growth chamber (Axyos Technologies e 2400 incubator). The ambient temperatures were set to mimic the diurnal summer range with a 12 hour cycle regulating light, humidity and temperature.

Growth chamber temperature and relative humidity (RH) parameters were set as follows:

- 19.1ºC and RH 63% at 9 am.
- 25.0 ºC and RH 47% at 9 pm.

The plants were randomly allocated to one of two groups:

- Group A: well watered (serving as control).
- Group B: watered only as deemed necessary (Test plants where drought conditions were simulated to stimulate potential stress responses within the plant).

Plants in Axyos chamber for a period of 54 days. (http://www.axyos.com/).

Figure 4 Group A plants following the growth chamber period. Most of these plants remained healthy and continued to flower throughout the 54 days during which they were regularly watered.

Comparative GC-MS Analysis of Hypochoeris radicata

Gas Chromatograph – Mass Spectrometry (GC-MS) metabolite profiling for semi-quantitative comparison of metabolite levels between the samples was performed.

H. radicatas samples tested included control batches where no known disease was associated with grazing and two test batches from properties with horses suffering from AS.

Three separate runs were prepared (complete results are provided in the supplement) .

Run 1

Control Batch: C

Test Batches: 1B and 2B (Both samples of mature *Hypochaeris radicata* associated with cases of AS).

Run 2

Separate plants from the following batches:

Control Batches: C, JF and 6

Test Batches: 1B and 2B mature *Hypochaeris radicata* associated with cases of AS).

Run 3

GC-MS performed on test and control plants from Growth Chamber Experiment (see above sub chapter).

Sample preparation

Representative samples of flower, leaf and stem were homogenized in the presence of liquid nitrogen. Approximately 30 mg of each test sample were weighed in Eppendorf tubes (2 mL) in the presence of liquid nitrogen. 500 micro litres of 100% MeOH along with 20 micro litres of the polar internal standard (0.2 mg/mL in water, sorbitol for TMS derivatisation) as a quantification standard was added to the homogenized sample and vortexed for 30 seconds. The mixture was extracted for 15 minutes at 70°C. The extract was then centrifuged at 13000 rpm for a further 10 minutes. The supernatant was then transferred to Eppendorf tubes (2 mL). 500 micro litres of MilliQ water was added to the pellet and vortex for 30 seconds and then centrifuged at 13000 rpm for a further 10 minutes. The supernatant was then combined with the first MeOH supernatant generated in the first extraction process. Fifty micro litre aliquots for each batch were dried *in vacuo*. One derivatisation methodology was carried out.

TMS Derivatisation: BSTFA with 1% TMCS

N,O-bis (Trimethylsilyl) trifluoroacetamide with Trimethylchlorosilane

The dry residues were re-dissolved and derivatised for 120 minutes at 37°C (in 10 micro litres of 30 mg/mL methoxyamine hydrochloride in pyridine) followed by treatment with 20 micro litres of BSTFA and 2.5 micro litres of a retention time standard mixture (0.029% (v/v) *n*-dodecane, *n*-pentadecane, *n*-nonadecane, *n*-docosane, *n*-octacosane, *n*-dotriacontane, *n*-hexatriacontane dissolved in pyridine) for 30 minutes. Sample volumes of 1 micro litres were injected onto the GC column using a hot needle technique.

Analytical instrumentation

The GC-MS system used comprised of a Gerstel 2.5.2 autosampler, a 7890A Agilent gas chromatograph and a 5975C Agilent quadrupole mass spectrometer (Agilent, Santa Clara, USA). The mass spectrometer was tuned according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43).

GC-MS method

Gas chromatography was performed on a 30 m VF-5MS column with 0.25 μm film thickness with a 10 m Integra guard column (Varian, Inc, Victoria, Australia). The injection temperature was set at 250°C, the MS transfer line at 280°C, the ion source adjusted to 250°C and the quadrupole at 150ºC. Helium was used as the carrier gas at a flow rate of 0.8 mL/min.

The analysis of TMS samples was performed under the following temperature program; start at injection 70°C, a hold for 1 minute, followed by a 7°C min^{-1} oven temperature ramp to 325°C and a final 6 minute heating at 325°C.

Mass spectra were recorded at 2scan s^{-1} with an *m/z* 50-600 scanning range. Both chromatograms and mass spectra were evaluated using the Chemstation program (Agilent, Santa Clara, USA). Mass spectra of eluting TMS compounds were identified using the commercial mass spectra library NIST (http://www.nist.gov), the public domain mass spectra library of Max-Planck-Institute for Plant Physiology, Golm, Germany (http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html) and the *in-house* Metabolomics Australia mass spectral library. All matching mass spectra were additionally verified by determination of the retention time by analysis of authentic standard substances. Resulting relative response ratios normalized per gram extracted fresh weight for each analysed metabolite were prepared as previously described (Roessner U, Luedemann A et al. 2001).

Results

Sample collection

Samples of *H. radicata* collected from properties containing horses affected with AS were potentially valuable in providing a positive test sample for analysis.

Plants from paddocks that house horses affected with AS were harvested in early autumn following a prolonged dry period, soils in these paddocks were unfertilised and of poor sandy quality.

Given that there is most likely a lag time of at least several weeks between plant exposure and development of signs (Huntington, Jeffcott et al. 1989) – field studies may be limited by temporal factors that differ from those that were present at the beginning of the disease process. Also sampling error factors may have resulted in false negative results if chosen plants were not typically affected. A wide range of plants were initially selected from affected properties including many potted mature plants for subsequent analysis. Subsequent problems resulting from either storage or testing methods rendered these samples no longer of any use to the study.

Growth Chamber experiment

Plants were harvested after fifty four days. During the trial most of the water deprived plants in Group B lost their leaves and appeared to die. After sporadic watering, several plants grew leaves again. At the completion of the study six of the ten droughted plants lost their rosettes and appeared to have died although the roots still appeared to be normal.

Seven of the ten plants in Group A that were well watered had healthy leaf structure, the remaining three all had leaves and stems that were dry and withered.

The group B droughted plants were only watered as deemed necessary for survival although this was difficult to judge since the large tap root allows *H. radicata* to survive prolonged less favourable conditions in a semi-dormant state. The result of this may have been that the plants may not have been sufficiently stressed and potentially leading to false negative results upon subsequent comparative analysis.

Leaves, flowers and stems were harvested. The GC-MS analysis of each of these was performed on the water restricted (test) and control plants. The full results are presented in the results supplement. Although a statistical analysis was not performed, the numerical differences between the test and controls did not appear as marked as those in the field samples.

Further studies would need to be performed to ascertain whether more severe stress is capable of producing greater differences in metabolites than was produced in this model. It is significant that there is little evidence of *H. radicata* being grown in growth chambers, with previous reports indicating a lack of success (Seddon and Belschner 1926; Pemberton and Caple 1980). This study was able to demonstrate successful growth and harvesting of *H. radicata* for 54 days under laboratory conditions, however it is not clear whether conditions simulating drought stress were produced.

Mycotoxin analysis

Semi-quantitative analyses for several known pasture based mycotoxins affecting livestock were conducted. The following results were established:

- Lolitrem B group: No analyte peaks were observed in the relevant chromatograms for any of the six samples analysed.

- Ergot-type alkaloids: No identifiable ergot alkaloid peaks were observed in the relevant chromatograms for any of the six samples analysed.

Although unidentified peaks were observed in both fluorescence detection and ultraviolet results, they were present in samples representing control and test samples.

As an extension to this task, the sample extracts from the lolitrem preparations were solvent-exchanged and run through this system also. The same result was arrived at: other unidentified peaks were observed (ulltraviolet and fluorescence detection) however they were present in samples representing both suspect sample and control sample groups.

Alkaloid screening – in a semi-quantitative screening for alkaloid presence -none of the six samples analysed showed any visible formation of precipitates or change in turbidity (JR Frelich and Marten 1973).

The ergot-type alkaloids represent a large range of mycotoxins affecting the nervous system of grazing livestock, these include paspalum staggers, phalaris staggers and more wider ranging toxicities associated with endophytic toxins. The most common and widely studied include the ergot-derived peptides, lysergic acid derivatives and tremorgens of the Loline group. Assays for all of these alkaloids in addition to more general alkaloid screening were negative in the test and control samples.

Although these tests have been broad in their scope, it is possible that other mycotoxins not detectable by standard alkaloid testing are present.

Given these findings it is unlikely that mycotoxins of the known alkaloid groups are responsible for the neurotoxicity observed in horses affected with AS. This finding reinforces those of Huntington et al who also proposed that mycotoxins may be a likely cause of AS (Huntington, Jeffcott et al. 1989).

Comparative GC-MS Analysis of Hypochoeris radicata[1]

A single injection (1 micro litre) of all three batches (C, 1B and 2B) were injected using GC-MS. The GC-MS total ion chromatogram (TIC) trace for Batch C, 1B and 2B is shown below (Figures 5 and 6 and Tables 1 and 2) showing differences in metabolite profile from control (juvenile) compared to stressed *H. radicata* samples.

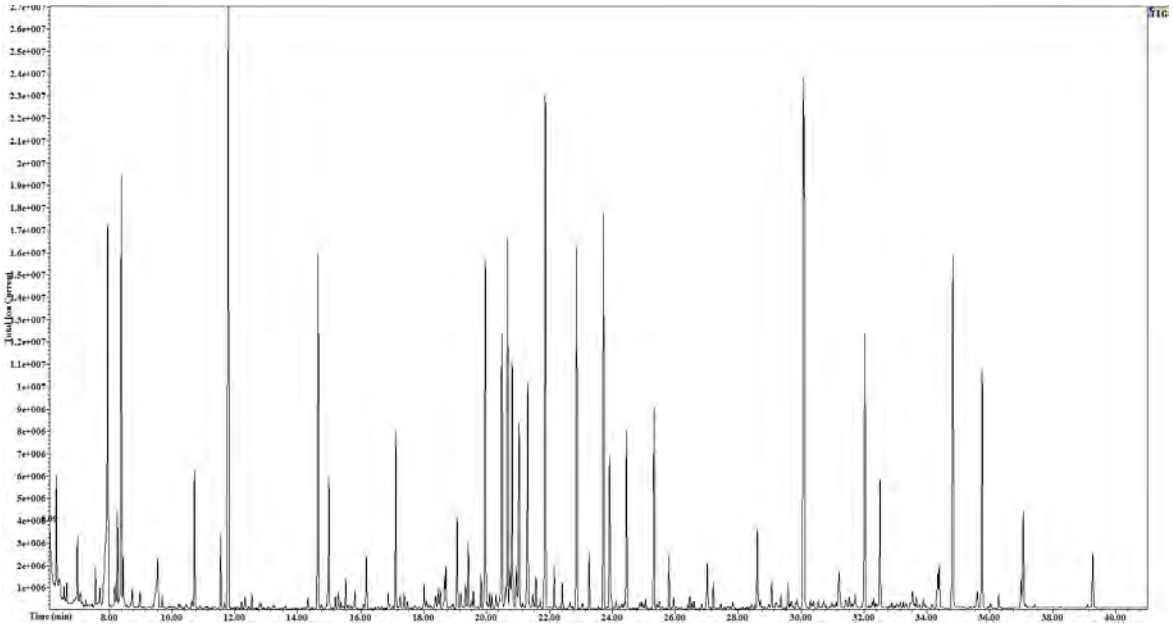

Figure 5 Run1 GC-MS TIC showing range of metabolites present in (control) Batch C.

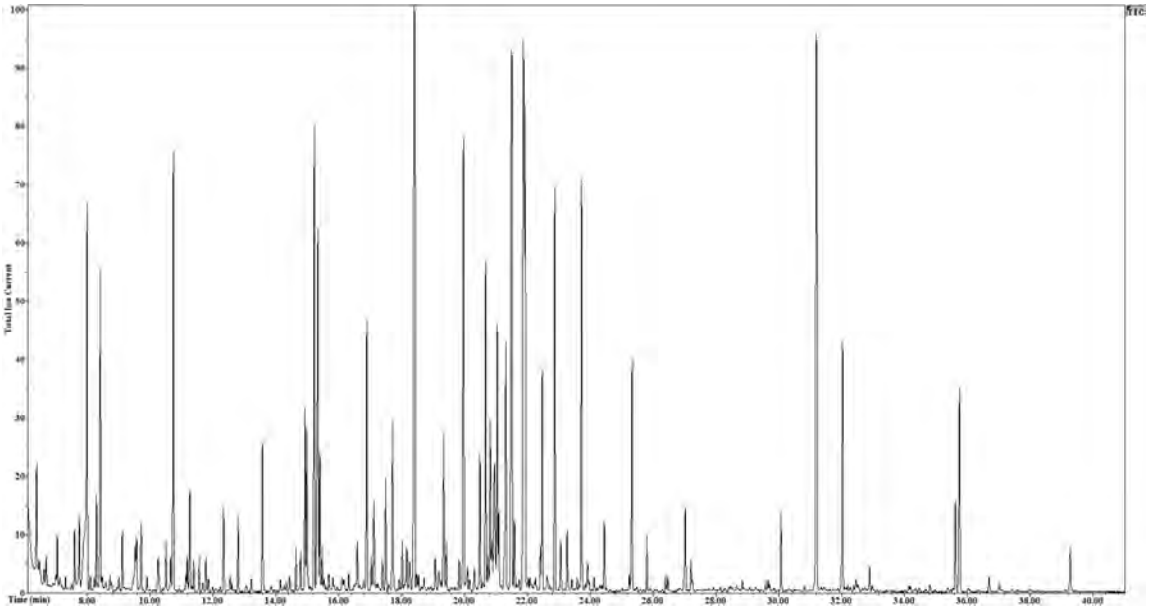

Figure 6 Run1 - GC-MS TIC showing range of metabolites present in Batch 1B.

[1] Complete results of Comparative GC-MS Analysis of *Hypochoeris radicata* are provided in results supplement including subsequent analyses of two test and 3 control batches.

Table1 Run1- GC-MS (abundance) analysis of three *H.radicata* batches C-1 (control) and 1B-1, 2B1 drought stressed plants from properties with horses affected with Australian Stringhalt. "Unknown" metabolites highlighted in green were not identified in the library.

Sample name	**C1_1**	**1B1_1**	**2B1_1**
Unknown_1	61.492	40.370	12.943
Alanine, DL- (2TMS)	66.241	321.725	83.735
Serine_2TMS	2.919	72.183	27.950
Ethanolamine	25.662	112.487	41.344
Phosphoric acid (3TMS)	440.497	639.984	874.905
Isoleucine	6.875	59.165	7.617
Threonine_2TMS	0.980	38.318	23.417
Aspartic acid	2.434	224.959	27.531
Malic acid, DL- (3TMS)	138.943	11.502	25.765
Threitol	2.551	33.663	2.528
Aspartic acid, L- (3TMS)	28.277	1162.060	15.844
Pyroglutamic acid, DL- (2TMS)	5.929	166.989	111.243
Butyric acid, 4-amino- (3TMS)	4.549	80.608	2.573
glutamic acid_2TMS	1.473	48.219	67.848
Threonic acid (4TMS)	28.062	0.000	0.631
Unknown_8	2.199	1.577	0.000
Glutamic acid, DL- (3TMS)	36.749	617.195	63.154
Tartaric acid (4TMS)	168.139	56.227	29.406
Xylose, D- (1MEOX) (4TMS)	8.741	65.583	3.585
Xylose, D- (1MEOX) (4TMS)	8.741	65.583	3.585
Asparagine_3TMS	0.000	184.814	0.922
Ribose	1.501	19.677	3.738
Unknown_9	1.822	28.118	0.000
Xylitol (5TMS)	13.835	11.894	1.497
Arabitol	18.876	1055.428	1071.004
Arabitol	8.098	297.092	288.719
Unknown_12	14.560	8.540	0.598
Unknown_13	17.627	0.000	0.000
Unknown_14	6.951	2.008	0.000
Keto-L-gluconic acid	25.741	25.019	0.000
Xylofuranose	43.975	34.277	21.315
Inositol, myo- (6TMS)	15.760	30.871	0.000
Inositol, L-chiro- (6TMS)	755.708	453.989	112.702
Unknown_24	245.652	22.502	0.592
Inositol, scyllo- (6TMS)	494.319	448.560	27.722
Inositol, myo- (6TMS)	271.479	228.994	32.161

Table 2 Run2 GC-MS abundance analysis of three H.radicata control batches C, 6F, JF and two test batches 1B and 2B (drought stressed plants from properties with horses affected with Australian Stringhalt). Selected metabolites and abundances (see supplement for complete list).

Sample Name	C	6F	JF	1B	2B
13C Sorbitol	93531.67	99208.67	80217.33	159481.7	84747.67
Valine	35193.33	162882.7	111815.3	939404.7	280494
Urea	33282	32637.67	26912.33	145829.7	47103
Benzoic acid	25517.67	18315	17567.67	27660	23174.33
Ethanolamine	61834.33	32957	44002.33	88295	19518
Phosphoric acid	2732839	4025288	3051141	4570270	1670156
Glycerol	111024	176981.3	267168	881531.3	331156.7
Isoleucine	14066.67	89895	51937.67	212353	114488.3
Proline	3469.5	4849.333	6290	13955.5	2901.667
Succinic acid	270177.7	251594.7	102889.3	79531.33	48431
Unknown 1	65998.33	64170.67	58798	63769.67	63039.67
Glycerate	6077441	2546465	3009595	147137	23790.33
Unknown 2	118854	223709.3	196735.7	159035.7	220957
Uracil	18023	16953	17206.67	50099.33	54817.5
Itaconate	9427	15068	15781.33	15669.67	11450.33
Fumarate	62266	66229.33	66229.33	170158.3	47114.33
Serine	85572.33	326087.7	169488	768812.7	439927
Unknown 16	137.3333	117.6667	123.3333	185014.7	85443.67
Glutamate	125102.3	307349	150205.3	1913570	1017065
Phenylalanine	9554	37197.67	23738	77846.33	38075.33
Tartaric acid	555215.3	1038490	560716.7	2040215	180753.7
Unknown 18	10885.33	53393	38565	20604.33	20340
Unknown 17	7042.333	11715	7342.333	82617	125.3333
Xylose	17577.67	69058	36070	118603	12391.67
Unknown 19	16536.33	44114.67	39239	158260.3	19571
Unknown 20	15883.33	66320	61429	44813	55587.33
Glutamine	410	99879.33	70555	773300.7	305674
Malate	1081215	3232398	1884362	548345	133975.7
Aspartate	112223.7	342803.3	223909.7	3115782	300355.3
Pyroglutamate	262084.3	726825	509897	4677350	2394915
GABA	65060.33	35495	91474.33	528903.3	21288
Gluconate	52807	14564	6922.333	327817.3	542905.7
Galactonate	54175.33	46691.67	30508.67	201425.7	899711.3
Scylloinositol	3549722	4587594	2840695	2434220	77870.67
L-chiroinositol	5880881	12737460	9873117	17700126	208031
Gulonic acid	2410.333	12691.67	6974	152605.7	806246
Saccharic acid	24292.33	13603.67	14996.33	43124	9279
2-oxoglutarate	34193	69954	2219	146.3333	177

It is apparent that the relative amount of unknown and known (eg. sugars, amino acids, TCA intermediates, fatty acids and organic acids) metabolites present in the control are numerically different in each batch. The known metabolites tentatively identified may play a significant role in the metabolism and thus secondary metabolism under stressed conditions, producing novel secondary metabolites. Further analysis of these preliminary results suggests that a large number of unknowns are present in the controls compared to the two stressed samples. As these are just preliminary results, a target library was generated from the control samples only.

While the list of chemical differences is extensive (see supplementary results for complete list in the Appendix) it is possible that some differences identified in the drought stressed plants may be responsible for toxic changes in *H.radicata.*

There are also some notable differences with several metabolites that are known to be neurotoxic. Given their known role in neurotoxicity it is intended to further investigate these differences in subsequent studies.

Marked increases were noted in the neurotransmitters aspartate and glutamate in the test batch B1 was noted compared to control plants in both run 1 and 2 (Figure 7). Both aspartate and glutamate are recognised in plant toxicities of horses grazing Russian Knapweeds or Yellow Star thistle (*Centaurea repens* and *Centaurea solistitial* respectively) (Roy, Peyton et al. 1995). These are both members of the Asteraceae family and the environmental factors of prolonged dry summers are similar to those of AS. Similarly to AS only horses have been reported to show clinical abnormalities following *C.repens* and *C.solstitial* ingestion.

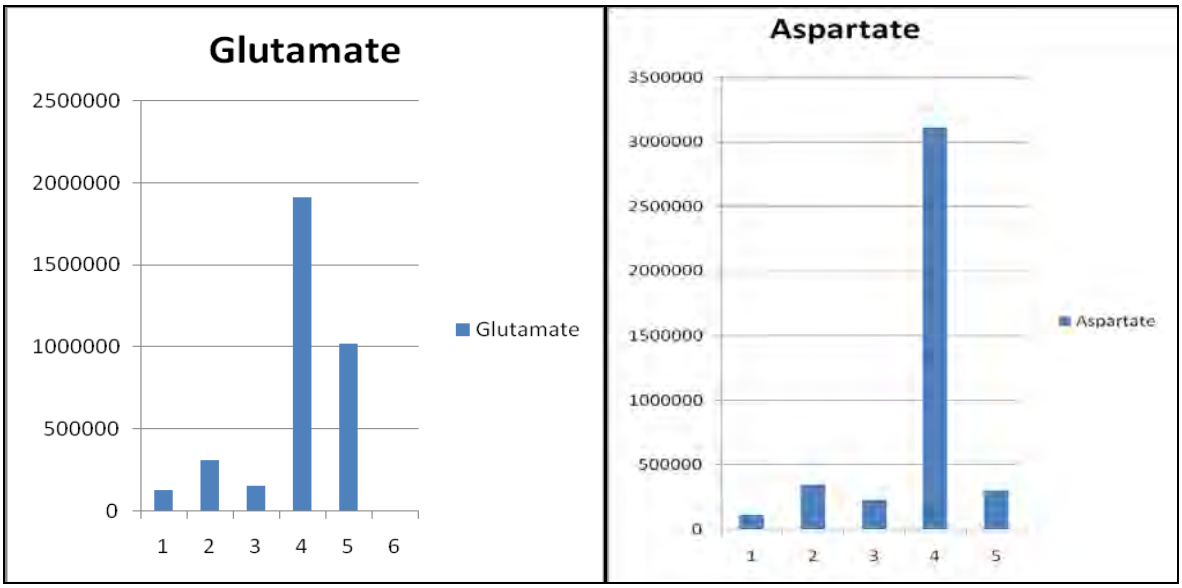

Figure 7 Run2 GC-MS Aspartate and Glutamate abundances. Control plants 1,2 and 3 . Batches 4 and 5 are from *H. radicata* on stringhalt affected properties(1B and 2B respectively).

Lathyrsm is a neurological condition resulting from the ingestion of *Lathyrus species* resulting in clinical abnormalities of horses similar to AS (Offord 2006). The toxin is believed to be a glutamate agonist known as beta-N-oxalylamino-L-alanine (BOAA) (Spencer, Roy et al. 1986) – this may be of potential importance with regard to investigating the role of this neurotransmitter in AS.

Despite these findings and past studies, the cause of the upper motor neuron dysfunction seen in AS cases is unclear. It has been suggested however that the distal axonopathy of the hindlimb nerves may

result in a reflex type of spasticity mediated via the muscle spindle receptors (Cahill, Goulden et al. 1985; Huntington, Seneque et al. 1991). It would be hoped that future investigations may identify the agent causing a peripheral neuropathy in addition to further studies of neurotransmitter function in cases of AS.

Inositol isomers have also been the focus of research into neurodegenerative conditions such as Parkinson's disease. These were examined and compared with relative differences between the batches. A study analysing metabonomically the feeding of *H.radicata* to mice found an increase in the levels of scyllo-inositol in the brain and liver (Domange, Canlet et al. 2008). There was little indication of such elevations in *H.radicata* from AS properties compared to control samples in this study.

Table 3 Inositol metabolites measured in the three H.radicata batches from Run1.

	C1_1	**1B1_1**	**2B1_1**
Inositol, myo- (6TMS)	15.760	30.871	0.000
Inositol, L-chiro- (6TMS)	755.708	453.989	112.702
Inositol, scyllo- (6TMS)	494.319	448.560	27.722

Of the unidentified metabolites (Figure 8), several compounds will be specifically targeted, these include the terpenoid group of chemicals. The sesquiterpene lactones are unstable compounds that are known to cause neurotoxicity in horses (Offord 2006). It is possible that they are precursors to neurotoxins in horses exposed to Yellow Star Thistle (Centaurea solstitialis) (Roy, Peyton et al. 1995). It is worth noting that a previous study has identified sesquiterpenes in *Hypochoeris radicata* (Bohlman and Bohlman 1980).

This was a pilot study with limited numbers of test and control samples and the distinct possibility of sampling error. It is therefore not possible to draw conclusions regarding differences between samples. However some obvious differences between control and drought stressed plants were observed, and the authors aim to investigate (and corroborate) these further.

GC-MS detects metabolites and matches them to several reference databases (eg. NIST). Identification of the unknown metabolites using a Natural Product Isolation approach could be conducted. *H.radicata* would be subjected to purification methodologies including extraction, solvent partitioning and trituration (eg. apolar, medium polar and polar solvents) followed by bench column chromatography (eg. silica, size exclusion chromatography and C18) then normal and/or reversed phase HPLC purification resulting in the isolation and purification of known or novel compounds. Compounds would ultimately be elucidated principally by 1D (^{1}H, ^{13}C and DEPT) and 2D NMR spectroscopy (gCOSY, gHSQCAD and gHMBC) and mass spectrometry (ESI-MS and GC-MS). Secondary techniques such as ultraviolet absorption spectroscopy (UV-Vis), fourier transform infrared spectroscopy (FT-IR) and optical rotation will also used as supporting evidence to support structures.

These methods are intended to be performed in anticipated future studies.

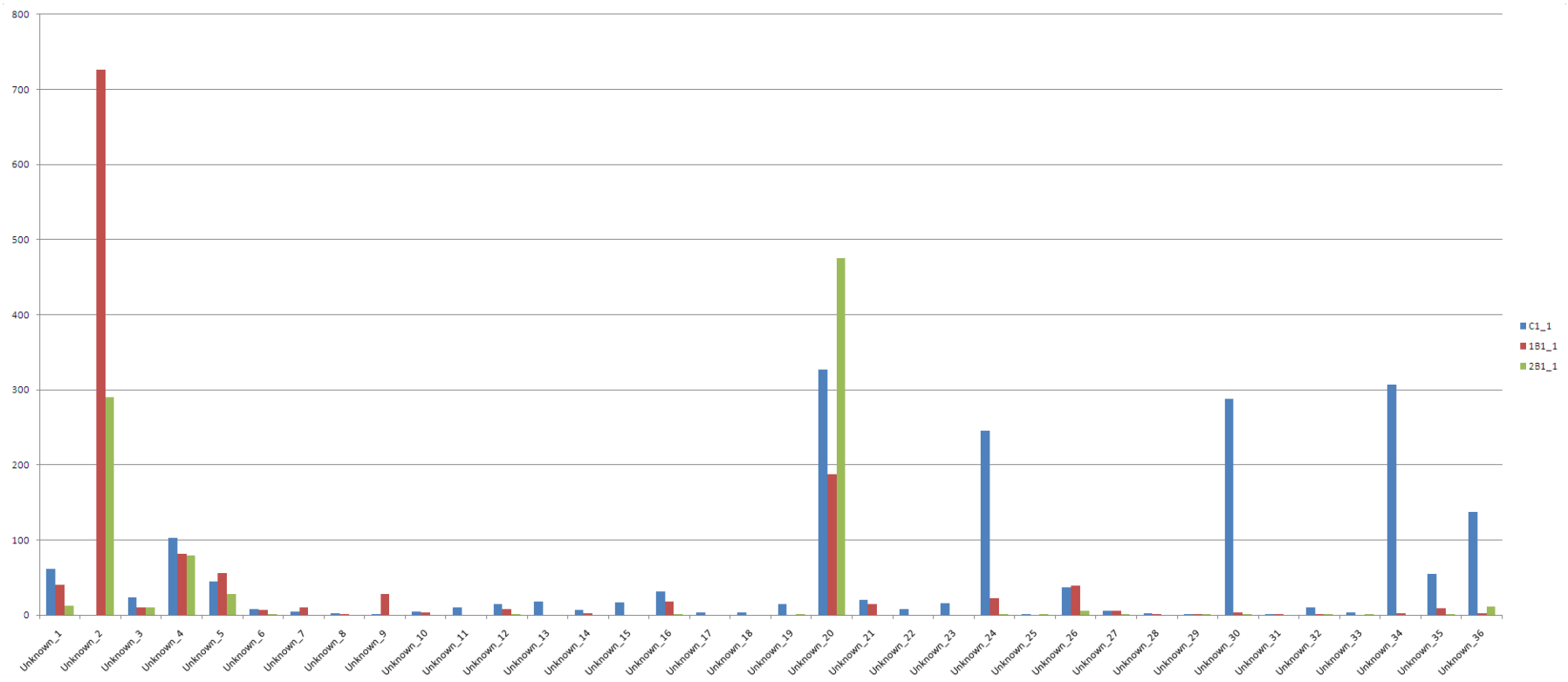

Figure 8 Column graph from run 1 showing levels of unknowns from Control sample C, and Test Batches 1B and 2B blue, maroon and olive columns respectively.

Implications

Horse welfare and health may be improved by the greater understanding of possible causes of Australian Stringhalt. Such understanding would be hoped to provide means to reduce the incidence of AS. It is also possible that identifying neurotoxic compounds associated with AS may assist the research into neurodegenerative conditions of people.

Further characterisation of the differences in metabolites of stressed *H. radicata* compared to control plants will provide additional valuable information.

The lack of evidence that known selected mycotoxic alkaloids are present in drought stressed *H. radicata* implicated in cases of AS is an important finding. These toxins have been suggested by many authors of significant AS studies (Pemberton and Caple 1980; Cahill, Goulden et al. 1985; Huntington, Jeffcott et al. 1989).

The feeding of mycotoxin binders to horses to prevent or treat stringhalt is not uncommon. This is based on mostly anecdotal recommendations to horse owners including many on internet based forums (author's observations).

It would appear given our findings and previous workers (Huntington, Jeffcott et al. 1989), that typical mycotoxic alkaloids responsible for grazing livestock derangements are unlikely to be the cause of AS. The rationale for feeding mycotoxin binders would therefore appear to have little substance.

Recommendations

The findings of our project, though preliminary in nature, strongly suggest that the weed *H. radicata* is able to produce a variety of metabolites in response to climatic stress and maturity.

Horse owners are often advised to feed a variety of supplements to treat or prevent stringhalt. Mycotoxin binders are not uncommonly recommended (author's observations). Our findings suggest that the practice of feeding mycotoxin (fungal toxin) binders may be unlikely to protect horses from developing AS.

Given these data, restricting access to pastures infested with *H. radicata* following prolonged dry weather conditions would likely be protective to horses from suffering from Australian Stringhalt.

Until a specific toxin is identified (whereby some form of binder or antidote may be possible) it would be advisable to avoid exposing horses to potentially contaminated pastures during certain periods.

There would be benefit in disseminating this information to horse owners as there is a need for peer reviewed information in the area of AS.

This work was a pilot study with an intention to further elucidate the differences in metabolites produced in drought stressed *H. radicata* and control plants.

Further work to be conducted would capitalise on the initial findings that this study has discovered could include the following:

- Prepare extracts of juvenile and mature (stressed) *H. radicata* and test them against various nerve cell cultures to monitor degeneration
- Consider a natural product isolation approach
- Solvent extract bulk samples (non-polar/polar solvents)
- Conduct bench column chromatography (eg. silica, C18) to fractionate extracts
- Test extracts in nerve cell cultures to track activity (bioassay guided approach)
- Isolate and purify potential novel sesquiterpene type compounds
- Determine structures of isolated compounds by NMR spectroscopy
- Carry out a complete structure elucidation by UV, FT-IR and MS techniques
- *InVitro* testing of isolated and purified compounds against various nerve cell cultures.

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Appendix: Supplementary Results GC-MS Analysis H radicata

Results

Table 1S GC-MS Response Sheet- complete listing Run 1

Sample name	C1_1	1B1_1	2B1_1
Fresh weight(g)	0.0344	0.0285	0.0292
ISTD_Sorbitol, D- (6TMS)	29.070	35.088	34.247
Lactic acid	85.911	36.487	54.881
Glycolic acid	12.427	3.470	13.303
Unknown_1	61.492	40.370	12.943
Alanine, DL- (2TMS)	66.241	321.725	83.735
Unknown_2	0.000	725.943	290.356
Unknown_3	23.480	10.215	10.301
Unknown_4	103.367	81.272	79.260
Malonic acid	0.987	4.393	0.394
Unknown_5	45.177	56.010	27.817
Urea	6.319	59.493	11.608
Benzoic acid (1TMS)	4.650	4.896	15.466
Serine_2TMS	2.919	72.183	27.950
Ethanolamine	25.662	112.487	41.344
Phosphoric acid (3TMS)	440.497	639.984	874.905
Isoleucine	6.875	59.165	7.617
Threonine_2TMS	0.980	38.318	23.417
Proline	5.489	456.516	59.669
Glycine	2.362	81.616	28.381
Succinic acid (2TMS)	40.452	15.105	8.240
Glyceric acid, DL- (3TMS)	709.162	18.337	2.289
Fumaric acid (2TMS)	19.422	9.561	19.222
Serine, DL- (3TMS)	25.284	158.660	10.433
Unknown_6	7.771	6.700	0.216
Threonine, DL- (3TMS)	1.917	24.401	2.146
Butanoic acid,2,4-dihydroxy-_3TMS	3.237	0.000	0.000
Aspartic acid	2.434	224.959	27.531
Malic acid, 2-methyl-, DL- (3TMS)	12.616	6.749	0.580
Malic acid, DL- (3TMS)	138.943	11.502	25.765
Threitol	2.551	33.663	2.528
Aspartic acid, L- (3TMS)	28.277	1162.060	15.844
Pyroglutamic acid, DL- (2TMS)	5.929	166.989	111.243
Butyric acid, 4-amino- (3TMS)	4.549	80.608	2.573
glutamic acid_2TMS	1.473	48.219	67.848

Threonic acid (4TMS)	28.062	0.000	0.631
2,4,6-Tri-tert.-butylbenzenethiol	8.199	6.259	6.148
Unknown_7	4.275	10.815	0.000
Threonic acid (4TMS)	17.165	6.831	0.133
Unknown_8	2.199	1.577	0.000
Glutaric acid, 2-oxo- (1MEOX) (2TMS)	7.856	0.560	1.005
Glutamic acid, DL- (3TMS)	36.749	617.195	63.154
Tartaric acid (4TMS)	168.139	56.227	29.406
Xylose, D- (1MEOX) (4TMS)	8.741	65.583	3.585
Xylose, D- (1MEOX) (4TMS)	8.741	65.583	3.585
Asparagine_3TMS	0.000	184.814	0.922
Ribose	1.501	19.677	3.738
Unknown_9	1.822	28.118	0.000
Xylitol (5TMS)	13.835	11.894	1.497
Unknown_10	5.308	3.357	0.000
Unknown_11	10.443	0.000	0.000
Arabitol	18.876	1055.428	1071.004
Arabitol	8.098	297.092	288.719
Unknown_12	14.560	8.540	0.598
Unknown_13	17.627	0.000	0.000
Unknown_14	6.951	2.008	0.000
Keto-L-gluconic acid	25.741	25.019	0.000
Xylofuranose	43.975	34.277	21.315
Unknown_15	17.297	0.000	0.000
glutamine_3TMS	0.640	244.398	3.988
Unknown_16	31.977	18.066	0.307
Unknown_17	3.844	0.000	0.000
Unknown_18	4.143	0.000	0.000
Shikimic acid (4TMS)	96.472	74.216	20.479
Citric acid (4TMS)	638.544	809.370	529.913
Unknown_19	14.291	0.000	0.795
Quinic acid, D(-)- (5TMS)	560.864	205.654	33.070
Fructose_MX1	326.472	119.399	475.836
Unknown_20	326.472	187.593	475.836
Unknown_21	20.941	14.486	0.000
Fructose, D (1MEOX) (5TMS)	228.042	119.399	475.836
Galactose_MX1	195.645	29.263	66.101
Galactose, D- (1MEOX) (5TMS)	118.818	136.998	42.402
Galactose_MX2	25.290	44.804	19.904
Inositol, myo- (6TMS)	15.760	30.871	0.000
Glucose, D- (1MEOX) (5TMS)	118.818	136.998	182.768
Galactonic acid-1,4-lactone	25.290	44.804	35.125
Mannitol, D- (6TMS)	14.248	939.609	210.162
Unknown_22	8.239	0.000	0.000

Inositol, L-chiro- (6TMS)	755.708	453.989	112.702
Unknown_23	16.129	0.000	0.000
Caffeic acid, trans- (3TMS)	4.207	0.878	0.000
Galactonic acid (6TMS)	15.681	10.943	2.747
Gluconic acid	15.681	85.495	51.461
Galactonic acid	3.425	119.854	67.788
Inositol, scyllo- (6TMS)	494.319	448.560	27.722
Saccharic acid (6TMS)	8.239	65.678	13.549
saccharic acid	8.239	65.678	13.549
Hexadecanoic acid, n- (1TMS)	62.810	51.235	41.367
Inositol, myo- (6TMS)	271.479	228.994	32.161
Unknown_24	245.652	22.502	0.592
Caffeic acid, trans- (3TMS)	314.729	86.662	12.741
Octadecanoic acid, n- (1TMS)	59.321	48.097	42.065
Unknown_25	0.980	0.000	0.608
Galactosylglycerol (6TMS)	3.151	2.606	0.546
Glucose-6-phosphate (1MEOX) (6TMS)	4.376	0.000	1.278
Unknown_26	36.866	39.336	5.798
Unknown_27	5.828	5.689	1.009
1-Benzylglucopyranoside	11.014	4.378	0.452
Unknown_28	3.032	0.725	0.000
Unknown_29	0.576	0.687	0.582
Unknown_30	288.341	3.439	1.310
Unknown_31	0.540	0.156	0.000
Unknown_32	10.344	0.534	0.145
Unknown_33	3.409	0.000	0.156
Sucrose	1242.549	123.854	586.080
Trehalose	62.736	1533.063	1388.736
Maltose, D- (1MEOX) (8TMS)	8.185	1.653	0.169
1-Monooctodecanoglycerol	10.657	1.441	1.241
Gentibiose_mx1	11.903	9.410	4.375
Unknown_34	307.302	2.917	0.000
Galactinol (9TMS)	1.349	0.000	0.000
Unknown_35	54.624	8.782	0.239
Quinic acid, 3-caffeoyl-, trans- (6TMS)	604.624	6.652	0.429
Raffinose (11TMS)	22.743	2.693	0.776
Unknown_36	137.453	2.958	11.057

All unknowns (normalized and highlighted in green) are shown in the Figure 8 column graph and clearly identifies varying amounts of unknowns identified from Batch C, 1B and 2B.

Table 2S GC-MS Response Sheet- complete listing Run 2

Sample Name	C	6F	JF	1B	2B
13C Sorbitol	93531.67	99208.67	80217.33	159481.7	84747.67
Valine	35193.33	162882.7	111815.3	939404.7	280494
Urea	33282	32637.67	26912.33	145829.7	47103
Benzoic acid	25517.67	18315	17567.67	27660	23174.33
Ethanolamine	61834.33	32957	44002.33	88295	19518
Phosphoric acid	2732839	4025288	3051141	4570270	1670156
Glycerol	111024	176981.3	267168	881531.3	331156.7
Isoleucine	14066.67	89895	51937.67	212353	114488.3
Proline	3469.5	4849.333	6290	13955.5	2901.667
Succinic acid	270177.7	251594.7	102889.3	79531.33	48431
Unknown 1	65998.33	64170.67	58798	63769.67	63039.67
Glycerate	6077441	2546465	3009595	147137	23790.33
Unknown 2	118854	223709.3	196735.7	159035.7	220957
Uracil	18023	16953	17206.67	50099.33	54817.5
Itaconate	9427	15068	15781.33	15669.67	11450.33
Fumarate	62266	66229.33	66229.33	170158.3	47114.33
Serine	85572.33	326087.7	169488	768812.7	439927
Unknown 3	6264	4942.333	5693	8309.667	8664.667
Unknown 4	16740.67	16528.67	12011	32798.67	100
Threonine	21214.67	68203.33	42416	616474.3	156805.7
Unknown 5	53980	53552.33	48582	58026.67	57929
Unknown 6	25380.33	14205.5	9348	9094	14794
Unknown 7	7622	15785	17154.67	5627.667	5674
Unknown 8	10793.33	12328.67	9147.667	8486	11733.67
3-amino-2-piperidin-2-one 2TMS	1826.667	2962.667	3013.333	118449	68789.67
2-methylmalic acid 3TMS	28152.33	23412.67	15382.67	78193	6754.667
Glutamine	410	99879.33	70555	773300.7	305674
Malate	1081215	3232398	1884362	548345	133975.7
Aspartate	112223.7	342803.3	223909.7	3115782	300355.3
Pyroglutamate	262084.3	726825	509897	4677350	2394915
GABA	65060.33	35495	91474.33	528903.3	21288
Unknown 9	27079.67	21753	21079.33	31061	23319
Erythronate	82692	45022.67	46505.67	35921.67	13052
Threonate	105868	114208	138614.3	60291.33	6357
Unknown 10	5192.5	13505.67	9257.667	83100.33	1808.333
2-oxoglutarate	34193	69954	2219	146.3333	177
Unknown 11	3103	47302	26255	11710.33	153
Unknown 12	77813	64156.67	48332	106509.7	102428
Unknown 13	23856.67	54045.33	58678.67	28776.33	45103
Unknown 14	31910.33	128060.3	153418.7	32065.67	45807.33
Unknown 15	23856.67	25258	31464	28776.33	45103
Unknown 16	137.3333	117.6667	123.3333	185014.7	85443.67
Glutamate	125102.3	307349	150205.3	1913570	1017065

Sample Name	C	6F	JF	1B	2B
Phenylalanine	9554	37197.67	23738	77846.33	38075.33
Tartaric acid	555215.3	1038490	560716.7	2040215	180753.7
Unknown 18	10885.33	53393	38565	20604.33	20340
Unknown 17	7042.333	11715	7342.333	82617	125.3333
Xylose	17577.67	69058	36070	118603	12391.67
Unknown 19	16536.33	44114.67	39239	158260.3	19571
Unknown 20	15883.33	66320	61429	44813	55587.33
Xylitol	113.6667	133	106	314564.3	8261.667
Unknown 21	2600.667	5024.333	3421.333	44835.33	1742
Arabitol	40589.67	28792	19401.67	8854998	5320797
Ribitol	34094	58341.67	78534.67	1338735	2436679
Unknown 23	24360	29302	42870	171116.7	134.3333
Unknown 24	13415.67	6096.333	6739.333	21442	124.6667
Ribonate	90190	94133.33	93509	402663.7	47343.67
2-keto-L-gluconic acid	7848.667	56323.67	32553	729565	231364.7
Shikimate	317718.3	645355	363822.3	169078	46949.33
Citrate	157268.7	420253	139303	357131.7	87919
Erythrose	1365	3963.333	2158.667	44590.67	15914.33
Quinate	3296629	1770055	1991382	889596	138841
Fructose MX1	262365.7	8353004	8002341	362768.7	66739.67
Unknown 25	33684.67	42900.67	19417.33	221860.7	1946
Fructose MX2	203263.3	6094972	5804869	257464.7	38809
Mannose	43504	52389.67	73416.67	52659.67	627545
Unknown 26	65716	77850.33	103545.7	131995	157242
Glucose MX1	503986	3485995	3869530	286402.7	271151.3
Glucose MX2	89462.33	697565.7	786560.3	54001.67	50383.67
Mannitol	71296.67	30761.33	23200.33	5561649	9958162
Tyrosine	10085.67	44690.33	34053.67	95462.33	122631.3
L-chiroinositol	5880881	12737460	9873117	17700126	208031
Gulonic acid	2410.333	12691.67	6974	152605.7	806246
Saccharic acid	24292.33	13603.67	14996.33	43124	9279
Gluconate	52807	14564	6922.333	327817.3	542905.7
Galactonate	54175.33	46691.67	30508.67	201425.7	899711.3
Scylloinositol	3549722	4587594	2840695	2434220	77870.67
Mucic acid	23367.67	44324.67	18853.67	1432518	98825
Hexadecanoate	231104.7	299386.3	288778.3	1265846	762855
Myoinositol	3046457	8335515	5879089	906009	296475.3
Transcaffeic acid	1032550	759427	848019.7	407925.7	178757.7
Tryptophan	2888	36419	13554.67	49718	39918
Unknown 27	118209.7	93325	76928.33	143408.3	153152.3
Octadecanoate	112724	129354.3	117556	308264	273567.3
Galactosylglycerol	73989.33	153976.3	253285.7	36231.33	13286.33
Unknown 28	43599.33	21929.67	22892.67	224492.3	110
Eicosanoate	8559	22335.67	14888	226591	184950.3

Sample Name	C	6F	JF	1B	2B
Unknown 29	37267	17974.33	18204.33	1838.667	124.3333
Hydroquinone-beta-glucopyranoside	60524	68288.67	40207.67	7040	114.6667
Sucrose	7599574	10751877	3884119	475580	254024.3
alpha,alpha-trehalose	73831.33	37318.67	50165.33	10146391	3914843
Betasitosterol	3910	4086.333	3132.667	5187	6771
Unknown 30	132	6727.667	6323	202981	253182.7

Table 3S Results of Run 3 Growth Chamber experiment GC-MS abundances of test plant tissues relative to control tissues. Leaf, stem and flower tissues of watered (control) *H. radicata* relative to water restricted (test) plant tissues.

	x-fold		% std err	x-fold		% std err	x-fold		% std err	x-fold		% std err	x-fold		% std err
	Control Flowers (CF)			Treated Flowers (TF)			Control Stems (CS)			Treated Stems (TS)			Control Leaves Dried (CLD)		
Amino Acids and Amines															
Alanine	1	±	0.299	1.044	±	0.353	1	±	0.317	0.473	±	0.453	1	±	0.351
Arginine	1	±	0.371	0.200	±	0.351	1	±	0.624	0.321	±	0.663	1	±	0.518
Aspartate	1	±	0.291	0.698	±	0.406	1	±	0.425	0.234	±	0.390	1	±	0.675
Asparagine	1	±	0.607	0.469	±	0.761	1	±	0.778	0.305	±	0.907	1	±	0.132
3-cyano-alanine	1	±	0.344	0.208	±	0.382	1	±	0.483	0.108	±	0.802	1	±	0.697
Beta-alanine	1	±	0.435	0.287	±	0.338	1	±	0.398	0.203	±	0.551	1	±	0.412
Ethanolamine	1	±	0.430	0.402	±	0.394	1	±	0.738	0.274	±	0.483	1	±	0.324
GABA	1	±	0.331	0.507	±	0.154	1	±	0.896	1.328	±	0.645	1	±	0.302
Glutamine	1	±	0.465	0.396	±	0.388	1	±	0.908	1.273	±	0.684	1	±	0.343
Glycine	1	±	0.361	0.432	±	0.324	1	±	0.780	0.177	±	0.683	1	±	0.277
Homoserine	1	±	0.405	0.980	±	0.684	1	±	0.387	0.127	±	0.560	1	±	0.840
Isoleucine	1	±	0.437	0.765	±	0.323	1	±	0.423	0.290	±	0.326	1	±	0.304
Ornithine	1	±	0.352	0.207	±	0.346	1	±	0.779	0.516	±	0.707	1	±	0.276
Proline	1	±	0.606	1.909	±	0.407	1	±	0.161	1.773	±	0.482	1	±	0.130
Pyroglutamate	1	±	0.383	0.309	±	0.311	1	±	0.382	0.185	±	0.420	1	±	0.421
Phenylalanine	1	±	0.432	0.493	±	0.177	1	±	0.459	0.108	±	0.382	1	±	0.656
Serine	1	±	0.411	0.543	±	0.106	1	±	0.723	0.346	±	0.255	1	±	0.384
Threonine	1	±	0.470	0.765	±	0.230	1	±	0.634	0.425	±	0.295	1	±	0.689
Tryptophan	1	±	0.396	0.884	±	0.387	1	±	0.817	0.367	±	0.674	1	±	0.403
Tyrosine	1	±	0.453	0.699	±	0.344	1	±	0.380	0.191	±	0.154	1	±	0.270
Uracil	1	±	0.407	1.206	±	0.577	1	±	0.132	0.221	±	0.150	1	±	0.145
Valine	1	±	0.519	0.620	±	0.197	1	±	0.578	0.322	±	0.343	1	±	0.503
Butyro-1,4-lactam	1	±	0.387	1.047	±	0.292	1	±	0.242	0.994	±	0.587	1	±	0.257
3-amino-2-piperidin-2-one	1	±	0.345	0.160	±	0.353	1	±	0.610	0.249	±	0.652	1	±	0.282

	x-fold		% std err	x-fold		% std err	x-fold		% std err	x-fold		% std err	x-fold		% std err
	Control Flowers (CF)			Treated Flowers (TF)			Control Stems (CS)			Treated Stems (TS)			Control Leaves Dried (CLD)		
Organic Acids															
2-Aminoadipate	1	±	0.432	0.490	±	0.269	1	±	0.479	0.108	±	0.323	1	±	0.220
2-aminobutyric acid	1	±	0.200	**0.412**	**±**	**0.125**	1	±	0.230	0.414	±	0.088	1	±	0.236
2-hydroxyglutaric acid	1	±	0.339	0.495	±	0.148	1	±	0.215	0.684	±	0.416	1	±	0.561
2-methylmalic acid	1	±	0.335	0.510	±	0.058	1	±	0.510	0.264	±	0.467	1	±	0.418
3-hydroxy benzoic acid	1	±	0.444	0.466	±	0.155	1	±	0.206	0.263	±	0.188	1	±	0.186
Aconitic acid	1	±	0.289	0.666	±	0.199	1	±	0.387	0.204	±	0.308	1	±	0.383
Benzoate	1	±	0.267	0.382	±	0.171	1	±	0.150	0.234	±	0.122	1	±	0.199
Malate	1	±	0.422	0.405	±	0.241	1	±	0.535	0.269	±	0.651	1	±	0.495
Tartaric acid	1	±	0.448	0.945	±	0.210	1	±	0.747	0.296	±	0.479	1	±	0.402
Citrate	1	±	0.477	0.482	±	0.272	1	±	0.678	0.129	±	0.397	1	±	0.316
Quinate	1	±	0.267	3.196	±	0.359	1	±	0.746	0.539	±	0.291	1	±	0.264
Transcaffeic acid	1	±	0.400	4.768	±	0.597	1	±	0.738	3.132	±	0.533	1	±	0.180
Caffeic acid	1	±	0.298	1.536	±	0.467	1	±	0.498	1.033	±	0.364	1	±	0.174
cis-3-caffeoylquinic acid	1	±	0.291	0.797	±	0.369	1	±	0.452	0.544	±	0.478	1	±	0.270
Fumarate	1	±	0.316	0.639	±	0.438	1	±	0.388	0.383	±	0.252	1	±	0.228
Glucarate	1	±	0.411	0.588	±	0.254	1	±	0.548	0.255	±	0.305	1	±	0.135
Gluconate	1	±	0.561	1.016	±	0.194	1	±	0.252	0.380	±	0.567	1	±	0.242
Glutamate	1	±	0.242	0.545	±	0.344	1	±	0.185	0.208	±	0.083	1	±	0.355
Glutaric acid	1	±	0.409	0.416	±	0.143	1	±	0.396	0.048	±	0.289	1	±	0.197
Glycerate	1	±	0.417	0.595	±	0.413	1	±	0.689	3.650	±	0.940	1	±	0.527
Glycerophosphoglycerol	1	±	0.490	0.504	±	0.191	1	±	0.313	1.190	±	0.573	1	±	0.169
Gulonic acid	1	±	0.476	0.283	±	0.269	1	±	0.398	0.227	±	0.332	1	±	0.187
Mucic acid	1	±	0.340	1.024	±	0.205	1	±	0.706	0.623	±	0.445	1	±	0.223
Saccharic acid	1	±	0.338	0.338	±	0.344	1	±	0.567	0.203	±	0.469	1	±	0.247
Shikimate	1	±	0.236	0.823	±	0.391	1	±	0.436	0.305	±	0.516	1	±	0.179
Succinate	1	±	0.144	**0.445**	**±**	**0.096**	1	±	0.483	0.723	±	0.435	1	±	0.243
Phosphate	1	±	0.495	1.452	±	0.436	1	±	0.639	0.482	±	0.533	1	±	0.482

	x-fold		% std err	x-fold		% std err	x-fold		% std err	x-fold		% std err	x-fold		% std err
	Control Flowers (CF)			Treated Flowers (TF)			Control Stems (CS)			Treated Stems (TS)			Control Leaves Dried (CLD)		
Fatty Acids															
Hexadecanoate	1	±	0.441	0.708	±	0.236	1	±	0.300	0.373	±	0.164	1	±	0.209
Octadecanoate	1	±	0.339	0.629	±	0.176	1	±	0.167	0.357	±	0.127	1	±	0.193
Sugars and Sugar Acids															
Arabitol	1	±	0.288	3.153	±	0.560	1	±	0.600	0.183	±	0.458	1	±	0.570
Arabinose	1	±	0.685	0.065	±	0.324	1	±	0.250	**0.029**	**±**	**0.189**	1	±	0.157
1,6-anhydroglucose	1	±	0.339	0.329	±	0.389	1	±	0.215	0.374	±	0.134	1	±	0.169
1-benzylpyranoside	1	±	0.449	0.440	±	0.334	1	±	0.601	0.190	±	0.320	1	±	0.235
Erythronate	1	±	0.311	0.643	±	0.071	1	±	0.558	1.146	±	0.603	1	±	0.420
Fructose	1	±	0.166	0.758	±	0.445	1	±	0.479	0.414	±	0.860	1	±	0.264
Glucose	1	±	0.176	0.791	±	0.471	1	±	0.425	0.369	±	0.880	1	±	0.156
Hydroquinone-beta-glucopyranoside	1	±	0.489	0.327	±	0.306	1	±	0.270	0.752	±	0.956	1	±	0.269
Galactitol	1	±	0.392	1.035	±	0.124	1	±	0.370	0.250	±	0.350	1	±	0.161
Galactonate	1	±	0.337	0.287	±	0.259	1	±	0.569	0.106	±	0.196	1	±	0.289
Galactosylglycerol	1	±	0.507	0.584	±	0.270	1	±	0.380	**0.065**	**±**	**0.390**	1	±	0.342
Gentobiose	1	±	0.215	1.115	±	0.291	1	±	0.381	0.798	±	0.307	1	±	0.203
Gulose	1	±	0.476	0.377	±	0.180	1	±	0.594	0.886	±	0.186	1	±	0.340
L-chiroinositol	1	±	0.575	0.683	±	0.110	1	±	0.751	0.341	±	0.194	1	±	0.375
Maltose	1	±	0.383	0.572	±	0.291	1	±	0.215	0.259	±	0.399	1	±	0.207
Mannitol	1	±	0.356	1.368	±	0.410	1	±	0.614	1.353	±	0.873	1	±	0.419
Mannose	1	±	0.449	2.488	±	0.298	1	±	0.447	1.543	±	0.658	1	±	0.324
Salicylaldehyde-beta-D-glucoside	1	±	0.257	0.794	±	0.316	1	±	0.297	0.194	±	0.347	1	±	0.283
Myoinositol	1	±	0.297	0.382	±	0.444	1	±	0.472	0.070	±	0.592	1	±	0.203
Scylloinositol	1	±	0.413	0.723	±	0.174	1	±	0.533	0.316	±	0.293	1	±	0.249
Trehalose	1	±	0.378	1.021	±	0.400	1	±	0.385	0.195	±	0.405	1	±	0.216
Threitol	1	±	0.327	0.910	±	0.128	1	±	0.570	0.107	±	0.510	1	±	0.595
Pinitol	1	±	0.217	**0.101**	**±**	**0.153**	1	±	0.186	**0.111**	**±**	**0.153**	1	±	0.154
Ribonate	1	±	0.487	0.468	±	0.294	1	±	0.835	0.142	±	0.434	1	±	0.264
Threonate	1	±	0.519	0.407	±	0.192	1	±	0.652	0.496	±	0.713	1	±	0.478
Xylitol	1	±	0.411	0.545	±	0.360	1	±	0.670	0.116	±	0.467	1	±	0.279
Glycerol	1	±	0.653	0.169	±	0.132	1	±	0.159	**0.123**	**±**	**0.245**	1	±	0.168
Sucrose	1	±	0.164	0.658	±	0.241	1	±	0.506	1.121	±	0.835	1	±	0.616
Xylose	1	±	0.531	0.668	±	0.380	1	±	0.556	0.482	±	0.436	1	±	0.240
Sugars Phosphates															
Glycerol-3-P	1	±	0.310	**0.225**	**±**	**0.181**	1	±	0.095	**0.605**	**±**	**0.272**	1	±	0.255
Inositol-1-P	1	±	0.432	0.703	±	0.281	1	±	0.532	0.774	±	0.697	1	±	0.344

	x-fold		% std err	x-fold		% std err	x-fold		% std err	x-fold		% std err	x-fold		% std err
	Control Flowers (CF)			Treated Flowers (TF)			Control Stems (CS)			Treated Stems (TS)			Control Leaves Dried (CLD)		
Other															
Unknown 1	1	±	0.519	0.408	±	0.233	1	±	0.429	0.043	±	0.408	1	±	0.182
Unknown 2	1	±	0.318	0.433	±	0.187	1	±	0.221	0.481	±	0.166	1	±	0.162
Unknown 3	1	±	0.358	0.439	±	0.207	1	±	0.190	0.652	±	0.227	1	±	0.196
Unknown 4	1	±	0.269	0.543	±	0.116	1	±	0.201	0.284	±	0.188	1	±	0.261
Unknown 5	1	±	0.254	0.461	±	0.184	1	±	0.234	0.832	±	0.208	1	±	0.197
Unknown 6	1	±	0.275	0.479	±	0.147	1	±	0.243	0.850	±	0.201	1	±	0.208
Unknown 7	1	±	0.186	**0.328**	**±**	**0.150**	1	±	0.214	0.966	±	0.218	1	±	0.177
Unknown 8	1	±	0.261	0.570	±	0.213	1	±	0.435	0.214	±	0.403	1	±	0.089
Unknown 9	1	±	0.387	0.309	±	0.535	1	±	0.688	0.170	±	0.852	1	±	0.813
Unknown 10	1	±	0.357	0.239	±	0.205	1	±	0.164	0.152	±	0.600	1	±	0.156
Unknown 11	1	±	0.364	0.620	±	0.300	1	±	0.235	**0.196**	**±**	**0.351**	1	±	0.178
Unknown 12	1	±	0.481	0.189	±	0.232	1	±	0.270	0.231	±	0.435	1	±	0.305
Unknown 13	1	±	0.338	1.054	±	0.442	1	±	0.464	0.125	±	0.511	1	±	0.485
Unknown 14	1	±	0.326	0.858	±	0.426	1	±	0.679	0.705	±	0.325	1	±	0.223
Unknown 16	1	±	0.394	0.071	±	0.195	1	±	0.760	0.149	±	0.794	1	±	0.884
Unknown 17	1	±	0.309	0.477	±	0.290	1	±	0.468	0.580	±	0.383	1	±	0.281
Unknown 18	1	±	0.369	0.974	±	0.301	1	±	0.354	**0.341**	**±**	**0.240**	1	±	0.948
Unknown 19	1	±	0.354	0.731	±	0.217	1	±	0.342	0.515	±	0.414	1	±	0.157
Unknown 20	1	±	0.496	0.171	±	0.238	1	±	0.839	0.569	±	0.284	1	±	0.227
Unknown 21	1	±	0.272	0.628	±	0.425	1	±	0.525	0.650	±	0.498	1	±	0.191
Unknown 22	1	±	0.234	0.406	±	0.493	1	±	0.645	1.629	±	0.643	1	±	0.293
Unknown 23	1	±	0.406	0.516	±	0.199	1	±	0.731	0.307	±	0.227	1	±	0.202
Unknown 24	1	±	0.627	0.152	±	0.443	1	±	0.714	0.261	±	0.596	1	±	0.227

	x-fold		% std err	x-fold		% std err	x-fold		% std err	x-fold		% std err	x-fold		% std err
	Control Flowers (CF)			Treated Flowers (TF)			Control Stems (CS)			Treated Stems (TS)			Control Leaves Dried (CLD)		
Unknown 25	1	±	0.540	0.152	±	0.252	1	±	0.595	0.290	±	0.635	1	±	0.271
Unknown 26	1	±	0.442	0.522	±	0.367	1	±	0.909	0.306	±	0.597	1	±	0.318
Unknown 27	1	±	0.240	0.599	±	0.217	1	±	0.330	0.506	±	0.241	1	±	0.205
Unknown 28	1	±	0.618	0.761	±	0.326	1	±	0.313	0.347	±	0.339	1	±	0.228
Unknown 29	1	±	0.311	0.493	±	0.313	1	±	0.203	**0.083**	**±**	**0.177**	1	±	0.225
Unknown 30	1	±	0.511	1.255	±	0.345	1	±	0.353	0.473	±	0.559	1	±	0.484
Unknown 31	1	±	0.837	0.039	±	0.306	1	±	0.435	0.053	±	0.438	1	±	0.315
Unknown 32	1	±	0.349	0.540	±	0.207	1	±	0.299	**0.319**	**±**	**0.421**	1	±	0.165
Unknown 33	1	±	0.262	0.435	±	0.347	1	±	0.477	0.401	±	0.292	1	±	0.231
Unknown 34	1	±	0.267	1.763	±	0.327	1	±	0.291	1.561	±	0.355	1	±	0.328
Unknown 35	1	±	0.358	0.184	±	0.351	1	±	0.224	0.679	±	0.635	1	±	0.245
Unknown 36	1	±	0.391	0.214	±	0.284	1	±	0.228	0.804	±	0.647	1	±	0.229
Unknown 37	1	±	0.371	0.618	±	0.094	1	±	0.381	0.345	±	0.351	1	±	0.203
Unknown 38	1	±	0.356	0.585	±	0.236	1	±	0.246	0.322	±	0.271	1	±	0.223
Unknown 39	1	±	0.386	0.983	±	0.383	1	±	0.408	0.490	±	0.292	1	±	0.202
Unknown 40	1	±	0.404	1.564	±	0.258	1	±	0.202	**0.524**	**±**	**0.456**	1	±	0.570
Unknown 41	1	±	0.379	0.642	±	0.286	1	±	0.264	0.988	±	0.176	1	±	0.299
Unknown 42	1	±	0.423	0.595	±	0.239	1	±	0.274	**0.205**	**±**	**0.291**	1	±	0.144
Unknown 43	1	±	0.346	1.144	±	0.227	1	±	0.577	0.540	±	0.321	1	±	0.174
Unknown 44	1	±	0.336	0.659	±	0.118	1	±	0.268	**0.115**	**±**	**0.424**	1	±	0.217
Unknown 45	1	±	0.324	0.676	±	0.176	1	±	0.275	**1.668**	**±**	**0.330**	1	±	0.301
Unknown 47	1	±	0.323	0.529	±	0.349	1	±	0.204	0.027	±	0.256	1	±	0.206
Unknown 48	1	±	0.498	2.853	±	0.398	1	±	0.203	0.529	±	0.555	1	±	0.166
Unknown 49	1	±	0.366	0.385	±	0.208	1	±	0.216	**0.071**	**±**	**0.262**	1	±	0.265
Unknown 50	1	±	0.420	0.748	±	0.266	1	±	0.258	0.091	±	0.178	1	±	0.196
Unknown 51	1	±	0.772	0.154	±	0.239	1	±	0.270	0.086	±	0.561	1	±	0.307
Unknown 52	1	±	0.391	0.363	±	0.196	1	±	0.327	0.105	±	0.471	1	±	0.307
Unknown 53	1	±	0.361	0.198	±	0.098	1	±	0.142	**0.505**	**±**	**0.335**	1	±	0.248
Unknown 54	1	±	0.515	0.577	±	0.061	1	±	0.309	0.194	±	0.213	1	±	0.223
Unknown 55	1	±	0.544	0.407	±	0.252	1	±	0.289	0.110	±	0.544	1	±	0.221
Unknown 56	1	±	0.632	0.597	±	0.352	1	±	0.465	0.538	±	0.371	1	±	0.165
Unknown 57	1	±	0.412	1.131	±	0.296	1	±	0.457	0.230	±	0.316	1	±	0.247
Unknown 58	1	±	0.300	0.479	±	0.169	1	±	0.294	0.211	±	0.285	1	±	0.445
Unknown 59	1	±	0.458	0.539	±	0.380	1	±	0.455	0.574	±	0.991	1	±	0.418
Unknown 60	1	±	0.419	0.565	±	0.363	1	±	0.368	0.045	±	0.564	1	±	0.236
Unknown 61	1	±	0.484	0.648	±	0.317	1	±	0.227	0.032	±	0.353	1	±	0.501
Unknown 62	1	±	0.331	0.971	±	0.294	1	±	0.311	**0.446**	**±**	**0.294**	1	±	0.164
Unknown 63	1	±	0.471	2.006	±	0.537	1	±	0.481	1.816	±	0.755	1	±	0.449
Unknown 64	1	±	0.462	1.295	±	0.404	1	±	0.767	0.550	±	0.693	1	±	0.288
Urea	1	±	0.462	0.242	±	0.363	1	±	0.317	0.101	±	0.267	1	±	0.248

KEY
P **< 0.05 / (number of metabolites)**
P **< 0.05**

Investigation into the Cause of Australian Stringhalt

by Charles El-Hage

Publication No. 11/127

Australian Stringhalt (AS) is a debilitating condition of pasture based horses resulting in an abnormal gait and involuntary hyper (over) flexion of the hind limbs when attempting to walk. It occurs in individual horses or in outbreaks. Prolonged dry summers on poorer quality soil types with unimproved pasture are recognised risk factors. The condition has been documented for over 120 years in south-eastern Australia and has been documented in several continents with little progress in determining the exact cause.

There are several common factors that are well reported in outbreaks of AS, they include a strong association with the presence of the flatweed *Hypochaeris radicata*. Most cases occur in late summer/autumn following prolonged dry spells with affected paddocks being typically of poorer unfertilised soil types. Although a strong association between the disease and *H. radicata* is well recognised there has been little published work to investigate potential toxins within or associated with the weed.

Funding for this project was made available from the RIRDC and the University of Melbourne's Faculty of Veterinary Science, Department of Land and Food Resources and Metabonomics Australia, School of Botany.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our Horse R&D program, which aims to assist in developing the Australian horse industry and enhancing its export potential.

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