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Presence, impact and retention of *Synergistes jonesii* in 'problem' herds grazing leucaena

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Abstract

A study of 8 commercial cattle herds grazing leucaena was undertaken to determine (a) the efficacy of *in vitro Synergistes jonesii* inoculum (produced in an anaerobic fermenter) in degrading DHP; and (b) the persistence of the inoculum following a period grazing non-leucaena pastures.

Cattle were introduced to the leucaena pastures for an initial period varying from 17 to 71 days. Fourteen to 15 animals were then sampled for (a) urine and blood plasma to determine toxicity status as indicated by concentration of DHP; (b) faeces for estimation of diet composition; and (c) rumen fluid for detection of *S. jonesii* by nested PCR analysis. After a further 42-56 days, animals were resampled as before to confirm toxicity status and inoculated with the *in vitro S. jonesii* inoculum; the herds were then sampled a third time (42-60 days after inoculation depending on grazier availability) to test the effectiveness of the inoculum in degrading DHP.

Five of the herds were then removed from leucaena pastures for periods ranging from 80-120 days and returned to leucaena pastures for 21 days to check persistence of the inoculum as indicated by retention of capacity to degrade DHP.

It was concluded that while most herds showed some capability to degrade DHP, either from inoculation or from residual capability from previous exposure, they did not achieve the same rapid and complete DHP degradation reported in the 1980s. At that time, inoculation with rumen fluid containing *S. jonesii* caused rapid degradation of DHP to very low levels within days of inoculation. In the present trials, there was: (a) a very slow rate of degradation of DHP isomers on some properties prior to inoculation; (b) frequent occurrence of high levels of 2,3-DHP in urine indicating partial toxin degradation, both before and after inoculation; (c) a low incidence of detection of *S. jonesii* in rumen fluid after inoculation based on PCR amplification of 16s rDNA sequence of the type strain 78-1 thus indicating low populations in rumen fluid; (d) no evidence of DHP degradation on one of two properties tested for efficacy of inoculation; and (e) loss of protection from subclinical toxicity on some properties after <4 months on non-leucaena pastures.

Given the uncertainty, there is a need to assess the integrity of the supply chain for the *in vitro* source of *S jonesii* and to test if the in-vitro source has changed in composition and/or effectiveness relative to the original mixed inoculum. Nevertheless, it was concluded that the *in vitro* inoculum was at least partially effective and should continue to be used by graziers until improved sources of inoculum and/or inoculation methodologies are demonstrated.

Executive Summary

Leucaena leucocephala (leucaena) is a tropical leguminous forage tree; when planted with companion grasses, it forms a highly valuable and productive grazing system for animal production. However, it contains the toxic, non-protein amino acid mimosine. Post-ingestion, mimosine is rapidly degraded by plant enzymes and rumen microbes into 3-hydroxy-4(1H)-pyridone (3,4-DHP), and often the isomer 2,3-DHP, which are detrimental to animal health. In the 1980's, the rumen bacterium *Synergistes jonesii* was found to rapidly degrade 3,4-DHP into harmless by-products. Inoculation with *S. jonesii* was found to greatly increase ruminant animal performance on diets high in leucaena and the problem of toxicity was thought to be resolved. However, sampling of cattle grazing leucaena during 2003/2004 (B.NBP.0340) indicated that up to 50% of mobs tested were found to have high urinary DHP concentrations and therefore may have been experiencing subclinical toxicity. There is now concern about the toxicity protection status of cattle grazing leucaena. Possible factors contributing to the re-occurrence of toxicity may relate to ineffective inoculation procedures or reduced effectiveness and/or persistence of the current *in vitro* source of *S. jonesii* inoculum.

The objectives of this project were to investigate the factors affecting the presence, introduction, impact and retention of *S. jonesii* in commercial herds with problematic 'status' (with respect to their protection from DHP-induced depressions in productivity).

Methods

Eight properties were selected in southern Queensland that were thought to be not protected from DHP toxicity by the *S. jonesii* bacteria. A subset of 14-15 animals were then selected from each property for pre and post-inoculation (with *in vitro S. jonesii*) measurement of urinary and blood DHP levels, presence and population number of *S. jonesii* in rumen fluid (real-time and nested PCR analysis), and liveweight gain (LWG). Estimates of leucaena and grass forage on-offer were made at each sample time.

On 4 properties, 5 herds of 6-8 animals were then removed from leucaena pastures for 80-120 days, before being returned to leucaena pastures for 3 weeks in order to monitor the retention of 'protection' from DHP toxicity following a period off leucaena.

Sampling times were: 3-10 weeks after first introduction to leucaena to establish herd toxicity status (S1); 10-17 weeks after first grazing leucaena when herds were inoculated with *in vitro S. jonesii* (S2); and then 6-8 weeks following inoculation (S3) to ascertain success of inoculation. Rumen fluid was sampled while the cattle were off leucaena pastures (S4) while both rumen fluid and urine were sampled after the animals were returned to leucaena pastures (S5).

Pre and post-inoculation results

Urine samples at S1 (after the initial 3-10 weeks on leucaena) showed elevated (mean >100 µg/mL) levels of DHP at 7 of the 8 properties, indicating that these properties were experiencing incomplete degradation of the toxins. However, after a further 10-17 weeks on leucaena (S2), only 2 of the 8 properties still demonstrated continuing incomplete degradation despite continuing high levels of leucaena in the diet. Thus the efficacy of inoculation could be assessed on two properties only - inoculation eliminated toxicity on one property but failed on the other.

Cattle on the other 6 properties apparently acquired DHP degrading capability after 10-17 weeks grazing leucaena from either (1) a population of type strain *S. jonesii* that was below the detection

of the PCR tests, (2) a residual variant population of *S. jonesii*, present at populations below the level of detection by real-time PCR, or (3) from a population of DHP degrading bacteria not yet identified. The real-time PCR was unable to detect or enumerate the *S. jonesii* type strain in any of the rumen samples collected during the trial. A possible variant of S jonesii was present in a very small number of samples. The development of more sensitive molecular tools (e.g. mRNA PCR techniques) capable of detecting/monitoring populations of *S. jonesii* $<10^4$-10^5 cells/mL in rumen fluid is required to better understand the population dynamics of this bacterium in the rumen.

Retention of* S. jonesii *when cattle grazed on non-leucaena pastures

When cattle were moved off leucaena onto alternative pasture diets for 80-120 days, and then returned to leucaena pastures, high urinary toxin concentrations showed that herds could lose 'protection'. Only 3 of the 5 mobs (2 of 4 properties) retained capacity to degrade DHP after 80-120 days off leucaena. It was hypothesised that persistence of *S. jonesii* may be more affected by type of diet than by the time off leucaena i.e. the physical and chemical properties of the alternative diets, which dictate the rumen environment and microbial growing conditions, may affect the persistence of *S. jonesii*.

This finding is especially relevant to graziers utilising leucaena in southern Qld where cold temperatures and frosts mean that other pasture must be used to finish cattle during the winter. At that time cattle may be moved onto lush forage crops. Such a radical change in diet may be difficult to maintain carrier animals on leucaena pastures. Further study is required under controlled conditions, not possible in the present study, to determine the effects of alternative diets on *S. jonesii* populations in the rumen.

Conclusions and management recommendations for retaining* S. jonesii *in cattle herds

Despite the equivocal outcome of this research, some graziers with leucaena pastures, even those previously inoculated with the *in vitro* source of *S. jonesii*, continue to report suboptimal 'protection' and now reinoculate on an annual basis to ensure that their herds are protected.

The original work on leucaena toxicity (>25 years ago) provides a basis for understanding the significance of the current results. That work showed that inoculation with *S. jonesii* derived from rumen fluid lead to rapid and almost complete degradation of DHP with very low levels being excreted in urine, usually less than 50 µg/mL, even for animals consuming high (including 100%) leucaena diets. In this work, a higher threshold level of mean concentrations in urine of DHP >100 µg/mL was set as the criteria for defining incomplete degradation.

Given the uncertainty, there is a need to assess the integrity of the supply chain for the in-vitro source of S jonesii and to test if the in-vitro source has changed in composition and/or effectiveness relative to the original mixed inoculum. Nevertheless, it was concluded that the *in vitro* inoculum should continue to be used by graziers until improved sources of inoculum and better inoculation methodologies are available.

Pending further research, it is recommended that graziers incorporate the following practices into their leucaena toxicity management program:

(a) Continue using the *in vitro S. jonesii* inoculum until improved inoculation methodology and application procedures are demonstrated.

(b) Regularly (at least annually) test herd toxicity status particularly at the start of the growing season when there is an abundance of lush leucaena forage. Cattle herds should be grazed on leucaena for a minimum of 3-4 weeks prior to toxicity testing using the colorimetric urine test developed by University of Queensland;

(c) Retest herd toxicity status 4-6 weeks after inoculation to confirm toxicity status; and

(d) Minimise time animals spend off leucaena pastures with a preference for maintaining 'carrier' animals on leucaena year-round.

Contents

1 Background

1.1 Problem identification and justification

Leucaena (*Leucaena leucocephala*) is a highly productive tropical leguminous fodder tree. It is long-lived and drought tolerant and is recognised for its excellent nutritional characteristics. These productive traits consistently deliver excellent live-weight gains, superior to most other tropical forage systems (Shelton and Dalzell, 2007). However, leucaena does contain the toxic non-protein amino acid mimosine, which is degraded by the rumen bacterium *Synergistes jonesii* into harmless by-products (Jones, 1981). When *S jonesii* is not present in the rumen, mimosine and its primary rumen derivative dihydroxypyridone (DHP) cause several adverse symptoms including suppression of liveweight gain (Jones *et al*., 1976; Quirk *et al*., 1988).

When *S. jonesii* was introduced into Australian ruminants in the 1980s, it enabled inoculated animals to consume high dietary intakes of leucaena and achieve almost complete degradation of DHP, and therefore display superior productivity (Jones *et al.*, 1983; Quirk *et al.,* 1988; Pratchett *et al.,* 1991). When introduced to a herd, its ease of transmission led scientists and graziers to believe that a 'once off' introduction of *S. jonesii* to a cattle herd would provide long-term protection against leucaena toxicity.

However, in 2004, extensive herd testing across Queensland (44 herds) indicated that approximately 50% of mobs grazing leucaena had significant risk of sub-clinical toxicity as indicated by elevated levels of 3,4-DHP or 2,3-dihydroxypyridone (2,3-DHP) or both in urine (Dalzell *et al.* 2012). Of particular concern, was the apparent occurrence of high DHP concentrations in herds that had been previously inoculated with an *in vitro S. jonesii* inoculum produced in an anaerobic fermenter (Klieve *et al.* 2002). The longevity and efficacy of the *in vitro* inoculum containing *S. jonesii* is poorly understood and may be different to the *in vivo* inoculum used throughout northern Australia prior to 1995.

The aim of this study was to investigate the efficiency and persistence of the *in vitro* inoculum in commercial cattle herds. Such information is vital to ensure the future management of *S. jonesii* and the continuing high productivity of cattle with access to leucaena pastures. The study was conducted on-farm.

The research reported here addressed the topic entitled "Presence, impact and retention of *Synergistes jonesii* in 'problem' herds grazing leucaena".

2 Project objectives

At the completion of the project on 15 Sep 2010, the project will have:

1. Investigated the factors affecting the presence, introduction (where required), and impact of *Synergistes jonesii* in commercial herds with problematic 'status' (with respect to their protection from DHP-induced depressions in productivity).
2. Explored the link between *S. jonesii* and liveweight production in suspected "problem" herds.
3. Investigated the post-inoculation retention of *S. jonesii* in a subset of these herds under different pasture/fodder regimes.
4. Using these results and published information, provided recommendations for managing and monitoring the retention of *S. jonesii* in cattle herds.

3 Methodology

Eight commercial Queensland cattle properties were selected in southern Queensland with well-established dry-land leucaena-grass pasture used for breeding, backgrounding and fattening (Table 1). The animal ethics approval number received for the project was SLAFS/SAS/944/08/MLA. Ethics approvals and information sent to graziers are given in Appendices 2-4. Three properties had been inoculated in the previous 5 years by oral drenching with *in vitro* inoculum from the same source as used in this study (properties 4, 6 and 8) and one had retained what they believed to be *S. jonesii* carrier animals (property 7).

The 8 target herds/properties were subdivided into 2 categories: a) 4x “naive” herds/properties that had never been inoculated; and b) 4x “problem” herds/properties that had been inoculated in the past but appeared to have lost protection. A short list of 13 properties was developed by the research team from graziers that had just planted leucaena and those who suspected they had a leucaena toxicity problem. Properties were selected and allocated to the 2 categories described above and were ranked on the following criteria:

i) inoculation & liveweight gain history

ii) suitability of leucaena pastures

iii) availability of suitable animal handling infrastructure (yards, vet crush etc)

iv) location – accessibility and diversity of the study

v) potential for alternate crop/pasture production (e.g. oats) for use in Expt 2

vi) collaborative capacity of the graziers (willingness to commit resources to the trial and to abide by experimental design)

The top ranked 4 properties in each category were then visited and the herds tested for toxicity using a simplified version of the UQ colorimetric on-farm (or crush-side) urine test. This test excluded the acid hydrolysis step and therefore underestimated urine toxin concentration. If animals tested had urine samples that developed strong colour results then they were included in the study.

Twenty-five animals were selected from each property for monitoring during the experimental period. Herds were categorised as potentially experiencing subclinical toxicity when excreting DHP at mean concentrations exceeding a threshold of 100 µg/mL. This level was chosen following the survey results reported by Dalzell *et al*. (2012).

Note that Property #7 was not sampled at S1 but sampling at S2 confirmed the earlier crush-side testing that showed moderately elevated urine DHP concentrations. It was also included in the study because the grazier had diligently and accurately recorded animal live weight gain, provided additional geographical spread of study sites, and had elected to inoculate the herd using inoculated animals borrowed from a neighbour. Sampling continued at Property 6 beyond S1, despite the average urinary DHP concentration at this time being less than 100 µg/mL, as there were some animals among the 15 sampled that were excreting DHP >200 µg/mL.

On all properties there was enough accumulated leucaena/grass biomass, relative to stocking rate, to sustain adequate levels of feed intake and relatively high proportions (>30%) of leucaena in diet. Paddocks were assessed and those with low/moderate grass supply were preferentially selected to

ensure consistently high levels of leucaena intake throughout the trial. However the low level of grass availability did not compromise overall (leucaena + grass) forage supply.

Treatments, animal management and measurements

The aim was to investigate the efficiency and persistence of the *in vitro* inoculum in commercial cattle herds. The experiment was conducted during 2009. In the first phase (Efficacy of inoculation; Apr-Jul), cattle grazing leucaena pastures on 8 properties were monitored over time, with sub-groups on each property allocated to plus/minus inoculation with *in vitro S. jonesii* culture, using the recommended procedure for oral drenching with thawed culture obtained from DEEDI laboratories at Yeerongpilly (Klieve *et al.,* 2002). The second phase (Persistence of inoculum; May-Oct) used 4 of the 8 properties, with 5 mobs of 6-8 animals removed from leucaena pastures for 80-120 days, before being returned to leucaena pastures for 3 weeks in order to monitor the retention of 'protection' from DHP toxicity following a period off leucaena.

Sampling times were:

Efficacy of inoculation (2 April – 17 July 2009, varying with site):

S1: Initial sampling after 17-71 days grazing leucaena to determine if animals were experiencing subclinical toxicity.

S2: Second sampling and inoculation, 42-56 days after S1.

S3: Third sampling to measure success of inoculation, 42-60 days after inoculation at S2.

Persistence of inoculum (7 May – 5 October 2009, varying with site)

S4: Several sequential samples were taken to monitor decline in populations of *S. jonesii* after removal from leucaena (rumen fluid samples, for PCR analysis only, on 6-8 occasions over 80–120 days).

S5: Final sampling to measure persistence of inoculum 21 days after animals were returned to leucaena pastures.

These sampling times increased the duration of the periods pre- and post-inoculation compared to the original time-table. The reasons for the changed timetable related to logistical issues, the individual needs of graziers and weather considerations. However, the changes had the effect of improving aspects of the trial as it extended the pre-inoculation period, revealing unexpected changes in toxicity status of cattle.

Cattle management - Efficacy of inoculation

Whilst 25 animals on each property were initially selected for monitoring (except on property #2 (14 animals)), the urine, blood and rumen fluid measurements were performed on a randomly selected sub-set of 14-15 animals at each site.

Animals were mustered immediately prior to sampling. Urine (DHP), blood (DHP), faecal (% leucaena in diet) and rumen fluid samples were collected from the same sub-set of animals on each occasion. Rumen fluid samples were collected from all 25 trial animals (24 animals on property #1) at the completion of the trial. During sampling, live-weights for each trial animal were recorded. On the rare occasion an animal became agitated during the sample collection process, it was released to minimise stress and risk of injury.

During the trial, the inoculum was handled with great care to ensure its viability was not compromised in storage or transit. All bottles of *in vitro* inoculum used in the study were collected from DEEDI where they had been stored at -20C. Samples were placed on dry ice (-78C) and then stored at UQ in a -80C freezer prior to use in the trial. Where possible, bottles of different batches of inoculum were used when more than 1 bottle was required to inoculate a herd. Inoculum was transferred to the farms frozen on dry ice, thawed in the shade immediately prior to use, quickly placed in a modified hot water bottle from which air was excluded, mixed together (if >1 bottle was used) and then administered by oral drenching (100 mL/hd) to 10% of each herd as per current industry guidelines.

For properties #1, #2 and #7 (Table 1), one leucaena paddock was grazed for the entire trial. For properties #3, #4, #6 and #8, new leucaena paddocks of similar condition and type were grazed as forage availability in the original paddock declined. For property #5, a rotational grazing system was employed comprising 4 similar leucaena paddocks where animals were moved as pasture availability and quality declined. Paddock stocking rates were determined by the collaborating graziers and ranged from 0.43 AE/ha to 3.33 AE/ha.

Cattle management - Persistence of inoculum

After the S3 sampling, five groups of 8 animals (properties #1, #4, #5 and #8) were moved to alternative leucaena-free diets for periods ranging from 80 to 120 days (Table 1). Cattle grazed either grass or oats. On property #5, two herds of 8 pregnant heifers grazed either oats/grass pasture or a native grass pasture.

Populations of *S. jonesii* in rumen fluid were monitored weekly initially and then less frequently (S4). At the conclusion of the leucaena-free diet period, animals were re-introduced to leucaena pastures for a three-week period to assess the retention of capacity to degrade DHP isomers and then resampled (S5).

Pasture measurements

Descriptors (‘low’, ‘moderate’ or ‘high’ edible dry matter availability) were established for yield ranges of <500 kg/ha, 500-1500 kg/ha and >1500 kg/ha of edible forage respectively, based on visual assessment of the pasture being grazed at the time of sample collection and referenced to calibrated photo-standards (A. Radrizzani, unpublished data, Plates 1 and 2).

Urine and faeces collection, treatment and analysis

Urine samples were acidified at a ratio of 9.5 mL: 0.5 mL of concentrated hydrochloric acid (32% HCl) to prevent microbial degradation of DHP. Samples were stored on ice in the field and later refrigerated prior to analysis. Urine was analysed using High Performance Liquid Chromatography (HPLC) to determine concentrations of mimosine, 3,4-DHP and 2,3-DHP using the modified method of Dalzell *et al*. (2012) adapted from Tangendjaja and Wills (1980). Concentrations of mimosine, 3,4-DHP and 2,3-DHP were also determined using the upgraded colorimetric test recently developed by The University of Queensland.

Faecal samples were collected using the rectal grab method, kept cool and stored out of direct sunlight, and oven-dried at 65°C. Dried samples were ground to particle size <0.1 mm and were sent for delta carbon analysis at the Australian National University, Canberra using a micromass IsoChrom connected to an EQ-1110 Elemental CHN-O Analyser. The method for the determination of the proportion of C3 (leucaena) and C4 (tropical grass) material was based on the work of Jones *et al*. (1979).

Table 1 Description of properties, pastures, cattle and sampling regime used to study the efficacy and persistence of an *in vitro S. jonesii* inoculum

Property ID	Location	Breed and class of animal	Starting liveweight* (kg± SE)	Leucaena pasture	Non-leucaena pasture	Days grazing leucaena at S1, S2, S3	Duration off leucaena (days)	Previous inoculation history
1	Millmerran	Angus M/F† weaners	251 ± 2.4	<3 years and vigorous – minimal grass	Oats (Taipan)	45 87 129	111	No
2	Millmerran	Angus/Wagyu x M/F weaners	241 ± 4.9	<3 years and vigorous – minimal grass	n.a.	53 95 137	n.a.	No
3	Millmerran	Brahman & Santa x heifers	333 ± 3.7	<3 years and vigorous – minimal grass	n.a.	30 72 114	n.a.	No
4	Goondiwindi	Brahman x steers	285 ± 7.4	<5 years, vigorous with established grass - buffel, blue grass and bambatsi	Native grass (Blue grass)	17 73 121	120	Yes (oral drench)
5	Dalby	Angus pregnant heifers	450 ± 7.5	<5 years, vigorous with established buffel, grass, Rhodes and purple pigeon	Oats (Reil) or Couch and Rhodes grass	71 119 175	112	No‡
6	Wandoan	Charbray & Brahman x steers	370 ± 8.0	<5 years, vigorous with established buffel grass	n.a.	54 110 160	n.a.	Yes (oral drench)
7	Wallumbilla	Santa x & Charbray steers	406 ± 10.9	<5 years, vigorous with established buffel grass	n.a.	- 77 122	n.a.	Yes (‘carrier’ animals)
8	Murgon	Droughtmaster & Braford steers	340 ± 11.6	<5 years, vigorous with established grass - green panic and Rhodes grass	Oats (Culgoa II) Green Panic and Rhodes grass	35 83 143	80	Yes (oral drench)

*Mean liveweight ± standard error at S1; †M/F = male/female
‡ No attempt to introduce *S. jonesii,* although a bull was purchased (for breeding purposes) from a recently inoculated property
§liveweight measured by the grazier on the 06.01.09

Rumen fluid, collection treatment and analysis with PCR

Rumen fluid samples were collected from restrained animals via an orogastric stomach tube. A sub-sample of rumen fluid was transferred into a polypropylene tube, stored on ice before freezing at -80°C. Equipment used during collection was sterilised by submerging in a HALASEPT (Intervet International B.V., Boxmeer, Holland) solution for 20-30 minutes after each use and flushed thoroughly with clean water. Rumen fluid samples were analysed using two methods.

Real-time PCR

Rumen fluid samples were thawed and DNA isolated using a modified method of Yu and Morrison (2004). The following procedure was used to isolate microbial DNA from the rumen fluid (C. Davis and N. Nguyen, personal communication).

The DNA was diluted to 1:3; 1:10 and 1:30 prior to analysis with real-time PCR assays to reduce inhibition. The following primers used were designed from multiple alignments of *S. jonesii* 16S ribosomal gene sequences from databases at CSIRO. Primers used were analysed using primer express and were compared with sequences available from NCBI (National Centre for Biotechnology Information) to confirm specificity. Cycle conditions used were one cycle of 50ºC for 2 minutes and 95ºC for 10 minutes for the initial denaturation, and 40 cycles of 95ºC for 15 seconds and 63ºC for 1 minute to facilitate primer annealing and product prolongation.

Primers used in real-time PCR analysis.

Forward primer	Reverse primer	Size (bp)
GCAAGTCGAACGGGGATCAT (60F-CAT)	TCCGTTGTCCCCCTGTAACT (137R)	102
ACTACTGTTACTTGAGAGAGATCGA (998F)	CGCTCGTTGCGGGACTTG (1091R)	118

Nested PCR

Isolated DNA was diluted to 1:3; 1:10 and 1:30 prior to analysis. A nested PCR test was incorporated into the project to determine the presence of *S. jonesii* DNA in rumen fluid samples. The following primers were analysed using primer express and were compared with sequences available from NCBI to confirm specificity. The initial amplification was performed using the 60F-CAT/1275R primer. PCR was also performed using the addition of 1.5 mM $MgCl_2$ and platinum (Invitrogen, Carsbad, CA) and the following conditions: one cycle at 94°C for 3 minutes and 25 cycles of 94°C for 20 seconds, 63°C for 30 seconds and 70°C for 45 seconds. The PCR product was purified using the pre-sequencing clean-up protocol. For every 2 µl of PCR product, a volume of 1 µl was added. Specifically this volume contained 0.25 µl of CIP (Calf Intestinal Alkaline Phosphatase) (10U/µl), 0.125 µl of 2 x Exol (10U/µl) and 0.625 µl of 5 x CS buffer. The sample was incubated at 37°C for 20 minutes and then at 80°C for 20 minutes to destroy enzyme activity and facilitate its use as a template for the second PCR. The primer pair 193F/1039R was incorporated for the second round of amplification. Similar PCR conditions described previously were employed and applied with the increase of cycles to 35 at 94°C for 20 seconds, 63°C for 30 seconds and 70°C for 45 seconds. All PCR products were analysed by running 2% agarose gels containing ethidium bromide and visualising for a single specific band product.

(A) 'High' availability of leucaena dry matter (>1500 kg/ha)

Property #1

Property #1

(B) 'Moderate' availability of leucaena dry matter (500-1500 kg/ha)

Property #6

Property #6

(C) 'Low' availability of leucaena dry matter (<500 kg/ha)

Property #3

Property #1

Plate 1 (A) 'High' availability of leucaena dry matter (>1500 kg/ha); (B) 'Moderate' availability of leucaena dry matter (500-1500 kg/ha) and; (C) 'Low' availability of leucaena dry matter (<500 kg/ha).

(A) 'High' availability of grass dry matter (>1500 kg/ha)

Property #6

Property #5

(B) 'Moderate' availability of grass dry matter (500-1500 kg/ha)

Property #4

Property #6

(C) 'Low' availability of grass dry matter (<500 kg/ha)

Property #8

Property #1

Plate 2 High' availability of grass dry matter (>1500 kg/ha); (B) 'Moderate' availability of grass dry matter (500-1500 kg/ha) and; (C) 'Low' availability of grass dry matter (<500 kg/ha).

Primers used in nested PCR analysis.

Forward primer	Reverse primer
GCAAGTCGAACGGGGATCAT (60F-CAT)	1275R (not available)
TAAAAGGAGCGATCCGGTAACA (193F)	CCATGCAGCACCTGTTCTAC (1039R)

Statistical analysis

Data were analysed using Microsoft Excel and Minitab 15 (2007, Minitab Inc., State College, Pennsylvania, USA). The mean urinary concentrations of 3,4-DHP and 2,3-DHP were analysed in both a raw and log transformed state. One-way ANOVA tests, one and two sample t-tests and descriptive statistics were used to test the data and compare sample periods. One tail t-tests were used to test whether DHP was > 100 µg/mL. Power and sample size analysis (one sample t-test) tests set at the universal power (0.8) were used to determine the number of samples required to accurately determine herd toxicity status. Descriptive statistics were used to summarise blood 3,4-DHP and 2,3-DHP toxin levels for each period. Delta carbon dietary composition data were presented as a percentage of leucaena in diet with summary statistics tabulated.

4 Results and discussion

Results

Pasture and diet composition

Leucaena on-offer varied greatly among properties during the trial. At the end of the conditioning period (3-10 weeks), all properties had either ‘moderate’ or ‘high’ levels of leucaena on-offer to animals (Table 2). After an additional 6-8 weeks grazing, availability of edible leucaena dry matter had decreased but was ‘moderate’ or ‘high’ on all properties except property #1 where it was ‘low’.

During the post-inoculation period, and when animals were returned to leucaena pastures after removal for 3 weeks, leucaena dry matter availability ranged from ‘low’ to ‘high’ (Tables 2 and 7).

Although leucaena on-offer varied among properties and declined with time, leucaena diet selection levels generally remained sufficient (>25% of diet at S3) to test the efficacy of inoculation on all properties, with the exception of property #7.

At the conclusion of the initial grazing period (S1), all properties except #7 (not measured) had average dietary leucaena levels ranging from 30 to 69% (Table 3). At the time of inoculation (S2), dietary composition on 6 properties ranged from 35 to 74% but had dropped to low levels of 18 and 14% on properties #4 and #7 respectively. Six to 8 weeks following inoculation (S3), the percentages of leucaena in diet on properties ranged from 26-70% except property #7 which continued to demonstrate low levels of leucaena in diet (14%). For cattle in the 5 herds that were moved to alternative pastures, their diets contained 29 to 64% leucaena on return to leucaena pasture.

Urinary DHP concentrations

Urinary DHP was measured by both HPLC and the colorimetric urine test kit approach. However, it was found that the colorimetric test could not accurately quantify concentrations of the DHP isomers when both were present in mixtures in the urine samples. This was due to the broad overlapping absorbance peaks for the chromophores the isomers produced with the ferric chloride reagent. Therefore it was concluded that, while the colorimetric test was useful as a qualitative test for the presence or absence of DHP isomers in urine samples, it could not be used as a quantitative diagnostic tool to determine the toxicity status of cattle herds grazing leucaena. By contrast, the HPLC method was very accurate and could definitively measure the concentrations of both isomers of DHP in the urine samples. All subsequent discussion regarding the efficacy of the *in vitro* inoculum is based upon the HPLC results.

Urinary total DHP (3,4-DHP plus 2,3-DHP) concentrations were highly variable within herds (Figure 2).Coefficients of variation ranged from 39 to 351% for 3,4-DHP and from 52 to 283% for 2,3-DHP; standard deviations for total DHP increased linearly with mean DHP concentration (Figure 3).

At the initial sampling, DHP excretions were significantly greater than the threshold concentration of 100 µg/ml on all properties except #6. Property #7 was not sampled. Unexpectedly, at S2 (prior to inoculation), urine DHP concentrations had dropped below the threshold level on all properties except #2 and #3. This meant that only these 2 properties could be used to test the efficacy of the inoculum at S3. After inoculation, DHP concentrations at S3 fell below 100 µg/mL on property #3 but not on property #2 (Table 3).

When the 5 cattle herds, all with low DHP levels at S2, that were removed from leucaena pastures for periods of 80-120 days, were returned to leucaena pastures for 3 weeks (Table 1), 2 herds (properties #1 and #8) showed high DHP levels above the threshold level while 3 herds (properties #4 and #5), were below the threshold level (Table 3).

Among properties that showed high levels of DHP, the percentage of 2,3-DHP varied from 0% to 99%. There were no trends with sampling time but some properties had consistently higher levels of 2,3-DHP than others e.g. property #5.

Variation in DHP concentrations in spot urine sample was poorly related to diet composition inferred from faecal analysis (Figure 4).

Blood plasma DHP concentrations

Concentrations of DHP in blood serum were generally very low apart from several sites at S1 (Table 4) and showed no significant correlation with DHP in corresponding urine samples. Concentrations ranged from non-detectable to 20.2 µg/mL for 3,4-DHP and from non-detectable to 16.2 µg/mL for 2,3-DHP. At the conclusion of the final 3 week leucaena grazing period in Phase 2, all properties showed total mean DHP concentration levels ≤13 µg/mL (Table 5).

Real-time and nested PCR analysis and effectiveness of the in vitro S. jonesii inoculum

The real-time PCR analysis method was unable to detect the presence of *S. jonesii* or enumerate populations of *S. jonesii* in the rumen fluid samples tested. It was assumed this was because the bacteria, if present, were in populations below the sensitivity of this method ($<10^4$-10^5 cells/mL). The more sensitive nested PCR analysis performed on the same samples revealed that 22 (6.1%) of 360 samples tested at S1 to S3 had detectable levels of *S. jonesii.* Only two of the 28 animals sampled after inoculation were positive to *S. jonesii* at S3 and none to the type strain. The

remaining samples either (a) did not have *S. jonesii* or; (b) populations below the level of detection by the nested PCR ($<10^4$-10^5cells/mL).

After the 5 herds were removed from the leucaena pastures, only 6 of the 376 samples collected during S4 (non-leucaena pastures) and at S5 (leucaena pastures) had detectable levels of *S. jonesii.* All 6 samples were collected during the first 21 days of the leucaena-free grazing period. There was no continuity in the detection of *S. jonesii* in individual animals among sampling times after first detection. Of the 15 inoculated animals at S2 only three tested positive to *S. jonesii* and only one of these at more than one sampling time (property #5, oats herd).

Examples of Nested and Real-time PCR primers, standard curves and detection limits for *S. jonesii* are given in Appendix 5.

Retention of DHP degradation capability

Analysis of the urine DHP data demonstrated that only 3 of the 5 herds moved onto non-leucaena pastures (properties #4 and #5) retained the capacity to degrade both DHP isomers following their return to leucaena pastures. This finding demonstrated that herds can lose 'protection' against DHP toxicity after just 80-111 days off leucaena.

The herds grazing oats on properties #1 and #8 (for 111 and 80 days respectively) failed to retain the ability to degrade DHP. The dominant isomer in both urine and blood samples from cattle on property #1 was 3,4-DHP, while on property #8 was 2,3-DHP (Table 3).

Live weight gain

There were no significant differences in liveweight that could be attributed to the effect of inoculation or pre-existing *S. jonesii* populations (Table 6). Liveweight gains declined in the final period (S3) on all properties, except properties #4 and #6, consistent with the advancing season and declining quantity of forage and the associated increasing grazing pressure

Table 2 Pasture evaluation for each property based on visual assessment of the ability of pastures to meet animal intake using calibrated photo-standards (Plates 1 and 2).

		Pasture evaluation							
		Trial period and pasture availability (quantity 'on offer')							
		Pre-inoculation conditioning period				**Pre-inoculation toxicity period**		**Post-inoculation recovery period**	
		Leucaena		**Grass**		**Leucaena**	**Grass**	**Leucaena**	**Grass**
	#	**Start**	**Finish**	**Start**	**Finish**	**Finish**	**Finish**	**Finish**	**Finish**
Property ID	**1**	High	High	Low	Low	Low	Low	Low	Low
	2	High	High	Moderate	Low	High	Low	High	Low
	3	High	Moderate	Low	Low	Moderate†	Low	Low	Low
	4	High	Moderate	Moderate	Low	Moderate†	Moderate in rows – high in adjacent pdk	Moderate	Moderate – low
	5	High	High†	High	Moderate	Moderate	Moderate	Low – moderate†	Low
	6	High	Moderate	Moderate	Low	Moderate	Low	High†	Low in rows – high in adjacent paddock
	7	*	*	*	*	Moderate	Moderate	Low	Low
	8	High	Moderate	High	Moderate	Moderate	Low	Low	Low

* No sample taken

† herd grazing a new paddock

Table 3 Summary statistics for concentrations (µg/mL) of 3,4-DHP and 2,3-DHP in the urine of animals grazing leucaena at 4 sample times (S1 = preconditioning grazing; S2 = at inoculation with *S. jonesii*; and S3 = post-inoculation with *S. jonesii*; S4 = rumen fluid samples for PCR testing only; S5 = after 3 weeks grazing after period off leucaena).

Property ID	Sample period	Mean % leucaena in diet (±SE)	Mean 3,4-DHP (µg/mL ±SE)	Mean 2,3-DHP (µg/mL ±SE)	Total DHP (µg/mL ±SE)	Sig. >100 µg/mL (*P* value <0.05)
1	S1	66 ±1	775 ±139	27 ±4	802 ±140	*
	S2	35 ±2	32 ±7	1 ±1	34 ±8	ns
	S3	31 ±2	45 ±14	2 ±1	47 ±16	ns
	S5	64 ±0.5	950 ±132	183 ±55	1134 ±179	*
2	S1	30 ±3	491 ±78	224 ±66	716 ±113	*
	S2	69 ±1	521 ±148	19 ±6	540 ±152	*
	S3	69 ±2	310 ±56	44 ±13	354 ±60	*
3	S1	55 ±1	664 ±133	294 ±75	958 ±193	*
	S2	55 ±1	686 ±68	41 ±8	727 ±73	*
	S3	58 ±1	56 ±10	2 ±1	58 ±10	ns
4	S1	41 ±1	376± 69	272 ±53	648 ±111	*
	S2	18 ±1	78 ±18	39 ±7	116 ±23	ns
	S3	26 ±1	nd	nd	nd	ns
	S5	29 ±1	2 ±2	nd	2 ±2	ns
5	S1	63 ±2	8 ±3	1036 ±309	1045 ±310	*
	S2	70 ±1	16 ±3	146 ±43	162 ±44	ns
	S3	70 ±2	13 ±2	55 ±29	68 ±29	ns
	S5	33 ±11 & 42 ±2	nd & 10±2	27 ±14 & 65 ±18	27 ±14 & 75 ±19	ns
6	S1	36 ±2	27 ± 25	43 ± 15	70 ±31	ns
	S2	74 ±1	10 ±2	30 ± 12	40 ±13	ns
	S3	51 ±2	9 ±1	122 ± 31	131 ±31	ns
7	S1	-	-	-	-	-
	S2	14 ±1	47 ±32	87 ± 22	134 ±45	ns
	S3	14 ±1	11 ±7	1 ± 1	11 ±7	ns
8	S1	69 ±2	217 ±45	nd	217 ±45	*
	S2	72 ±2	37 ±21	1 ± 1	37 ±21	ns
	S3	41 ±2	6 ±4	1 ± 1	7 ±4	ns
	S5	45 ±3	118 ±36	490 ±126	609 ±139	*

* mean herd total DHP concentration >threshold concentration of 100 µg/mL (*P* value >0.05); - no samples collected: nd not detectable: ns not significant

Table 4 Summary statistics for concentrations (µg/ml) of 3,4-DHP and 2,3-DHP in the blood plasma of animals at 3 different sample times (S1 = preconditioning period grazing leucaena; S2 = period before inoculation with *S. jonesii*; and S3 = period post inoculation with *S. jonesii*).

		Sample period		3,4-DHP (µg/mL)		2,3-DHP (µg/mL)	
			n	Mean ± SE	Range	Mean ± SE	Range
Property ID	1	S1	14	9.1 ± 0.9	4.1 – 17.0	nd	nd
		S2	15	0.2 ± 0.1	nd – 0.8	nd	nd
		S3	14	0.2 ± 0.1	nd – 0.5	nd	nd
	2	S1	14	6.9 ± 0.8	2.4 – 12.7	2.2 ± 0.7	nd – 6.2
		S2	13	8.6 ± 0.8	3.6 – 16.1	nd	nd
		S3	14	4.6 ± 0.5	1.1 – 7.5	0.2 ± 0.2	nd – 3.1
	3	S1	13	11.1 ± 1.2	5.8 – 18.3	3.2 ± 0.6	nd – 6.9
		S2	15	5.7 ± 0.5	3.3 – 9.2	nd	nd
		S3	15	0.4 ± 0.1	0.1 – 1.1	nd	nd
	4	S1	15	1.8 ± 0.3	nd – 4.3	nd	nd
		S2	15	0.6 ± 0.2	nd – 2.8	nd	nd
		S3	13	nd	nd	nd	nd
	5	S1	13	nd	nd	4.7 ± 1.5	nd – 16.
		S2	15	0.4 ± 0.4	nd – 6.3	0.6 ± 0.3	2 nd – 3.7
		S3	15	nd	nd	nd	nd
	6	S1	14	0.4 ± 0.4	nd – 5.8	0.2 ± 0.1	nd – 1.5
		S2	14	nd	nd	nd	nd
		S3	14	1.4 ± 1.4	nd – 20.2	0.7 ± 0.5	nd – 5.2
	7	S1	-	-	-	-	
		S2	14	0.2 ± 0.1	nd – 1.5	nd	nd
		S3	14	nd	nd	nd	nd
	8	S1	15	1.5 ± 0.2	nd – 3.2	nd	nd
		S2	15	0.6 ± 0.2	nd – 2.6	nd	nd
		S3	14	nd	nd	nd	nd

- No samples collected; nd = Not detectable

Table 5 Summary statistics for concentrations (µg/mL) of DHP isomers in the blood of cattle 3-weeks after they returned to leucaena pastures following 3-4 months grazing grass-only or oats pastures.

Property ID	n	3,4-DHP		2,3-DHP	
		Mean ± SE	Range	Mean ± SE	Range
1	8	12.3 ± 1.1	9.5 - 18.6	0.3 ± 0.2	nd - 1.8
4	8	nd	nd	nd	nd
5 (Oats)	8	nd	nd	nd	nd
5 (Grass)	7	nd	nd	0.1 ± 0.1	nd - 1.0
8	8	0.6 ± 0.2	nd - 1.8	4.0 ± 2.2	nd - 23.6

nd Not detectable

Table 6 Liveweight gain summary for herds grazing leucaena pastures during each sample period (S1 = preconditioning period grazing leucaena; S2 = period before inoculation with *S. jonesii*; and S3 = period post inoculation with *S. jonesii*).

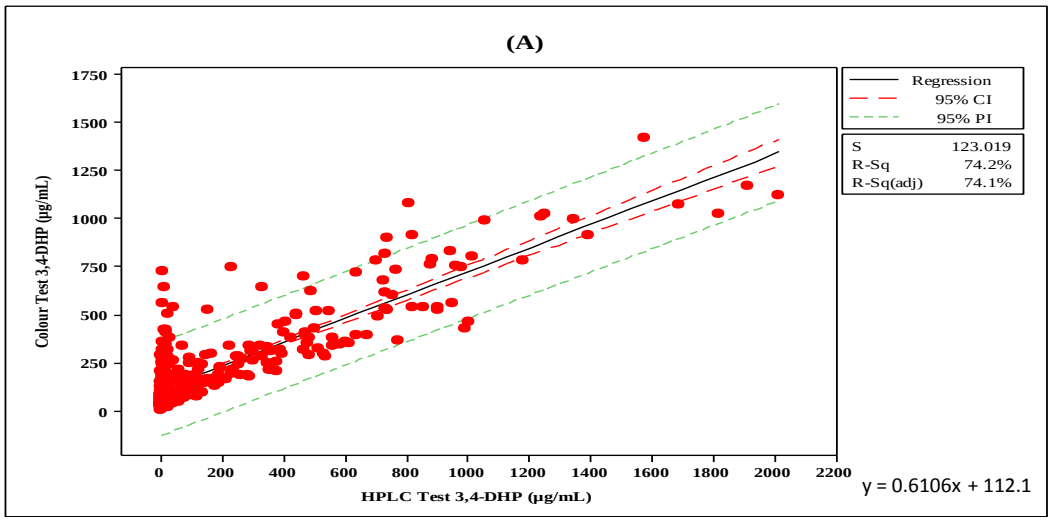
Property ID	Sample period	n	Mean liveweight gain ± SE (kg/hd/day)
1	S1	25	0.75 ± 0.047
	S2	25	0.89 ± 0.054
	S3	25	-0.48 ± 0.053
2	S1	14	0.93 ± 0.049
	S2	14	0.95 ± 0.046
	S3	14	0.15 ± 0.071
3	S1	29	*
	S2	29	0.31 ± 0.061
	S3	25	0.22 ± 0.084
4	S1	24	0.44 ± 0.056
	S2	24	0.76 ± 0.025
	S3	24	0.87 ± 0.030
5	S1	25	*
	S2	25	0.84 ± 0.044
	S3	25	0.22 ± 0.031
6	S1	29	*
	S2	29	0.61 ± 0.034
	S3	29	0.89 ± 0.034
7	S1	15	*
	S2	15	1.10 ± 0.056
	S3	15	0.71 ± 0.072
8	S1	16	0.75 ± 0.081
	S2	16	0.95 ± 0.054
	S3	16	0.83 ± 0.044

* No data available due to initial curfew weights confounding estimates of LWG.

Table 7 Visual assessment of dry matter availability of leucaena-grass pastures to which cattle were returned following 3-4 months grazing grass-only or oats pastures.

	Quantity of edible dry matter 'on offer'			
	Leucaena		Grass	
Property ID	**Start**	**Finish**	**Start**	**Finish**
1	High	High	Low	Low
4	Low	Low	Low	Low
5 (Oats Group)	High	High	Low	Low
5 (Grass Group)	High	High	Low	Low
8	Low	Low	Low	Low

Low = <500 kg/ha; Moderate = 500-1500 kg/ha; High = >1500 kg/ha



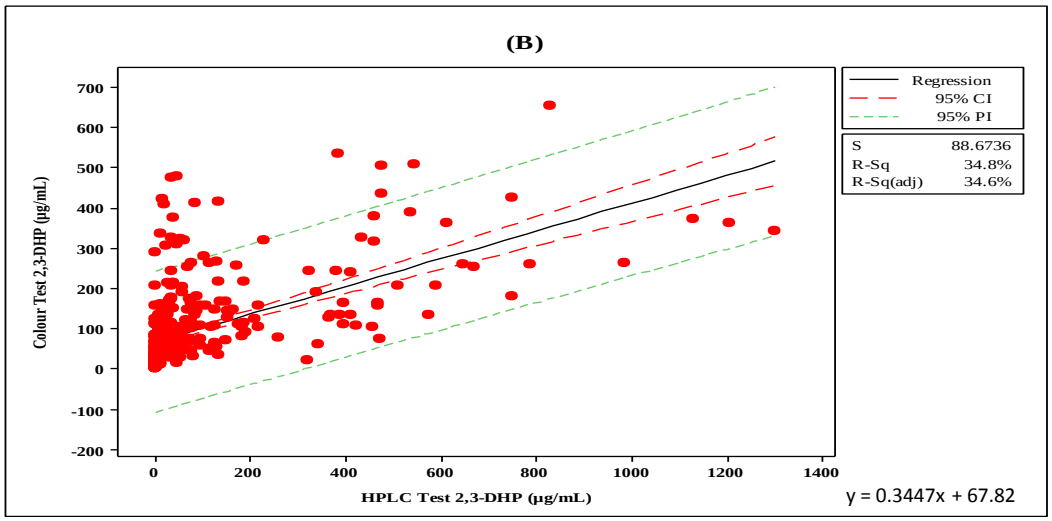


Figure 1 Regression analysis of paired data for urinary DHP excretion as detected by colorimetric and HPLC analysis where outliers have been omitted: (A) 3,4-DHP and (B) 2,3-DHP. (CI = Confidence interval; PI = Prediction interval; and S = Standard deviation of residuals and represents the average dispersion around the trend line)

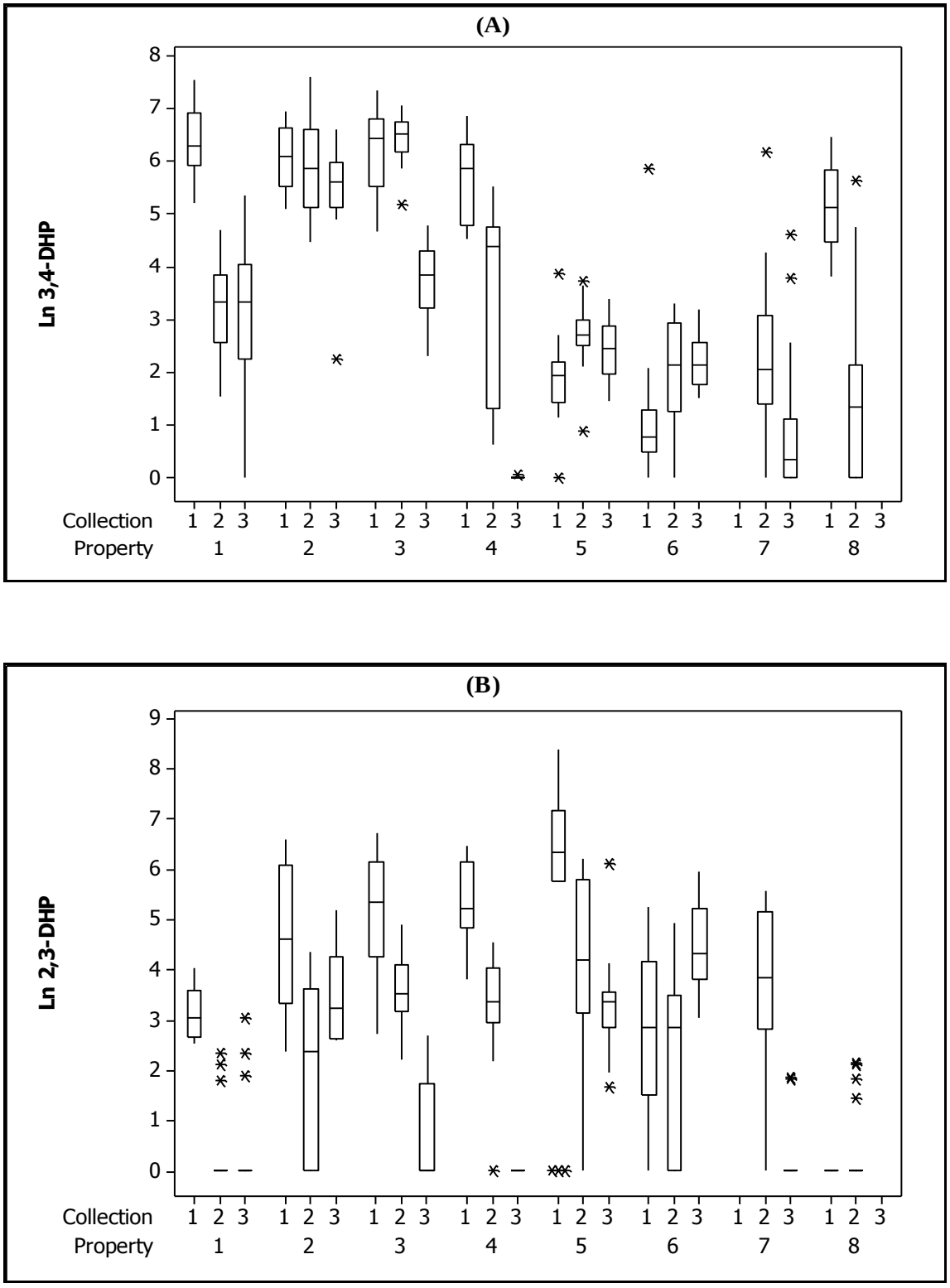


Figure 2 Variability in urine toxin excretion patterns. Log transformed (A) 3,4-DHP and (B) 2,3-DHP urinary excretion concentrations plotted at each sample date within properties #1-8 (* describes outliers).

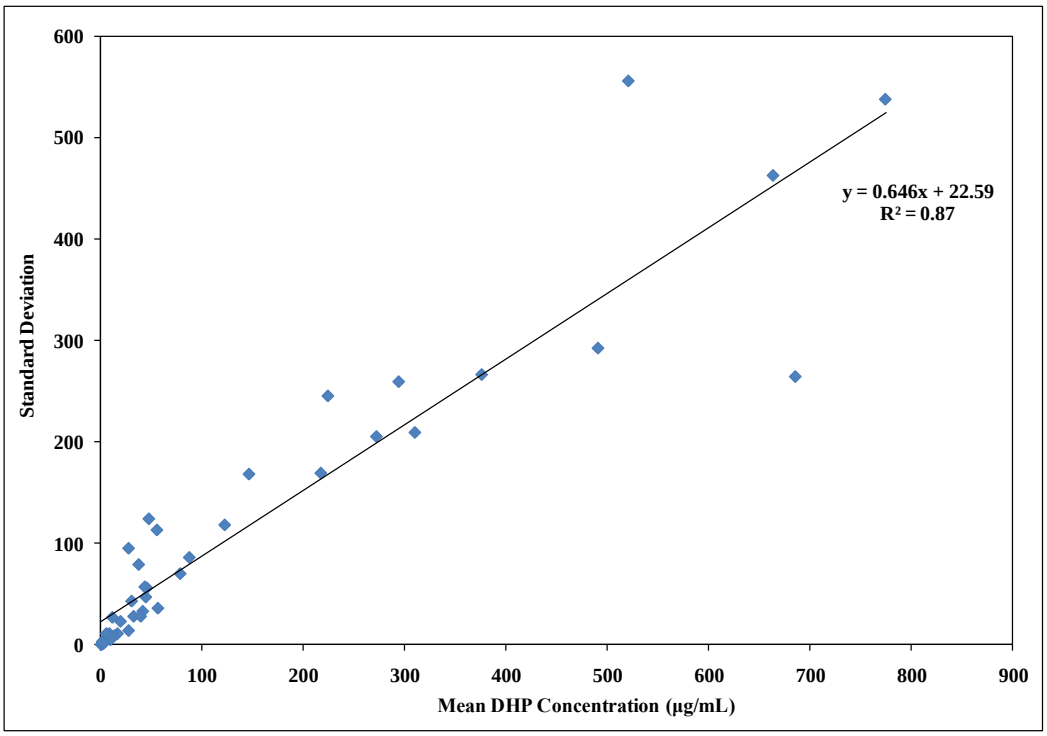


Figure 3 Average herd DHP concentration in urine plotted against herd standard deviation in urine concentration.

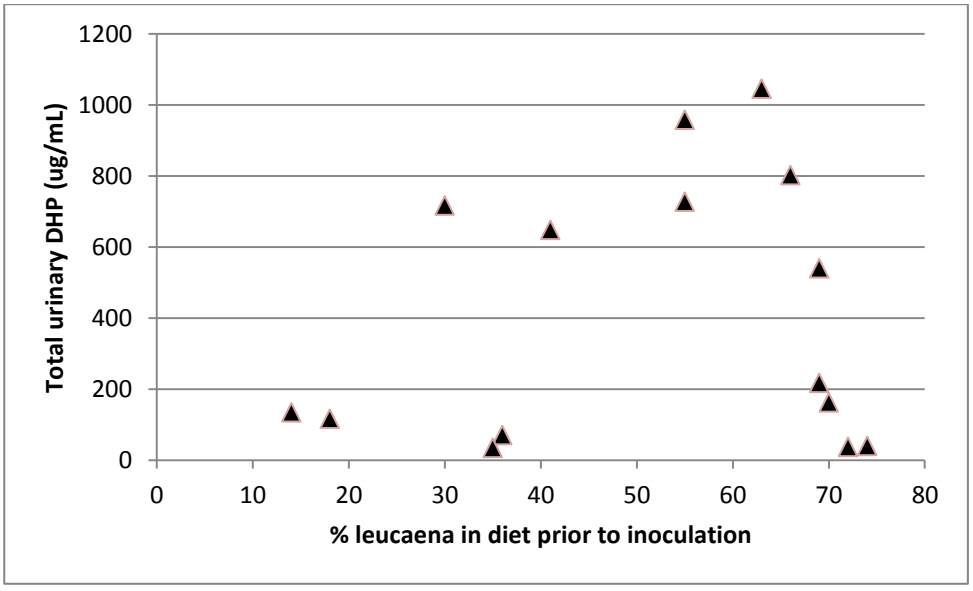


Figure 4 Plot of DHP concentration in spot samples of urine versus % leucaena in diet as determined by faecal analysis.

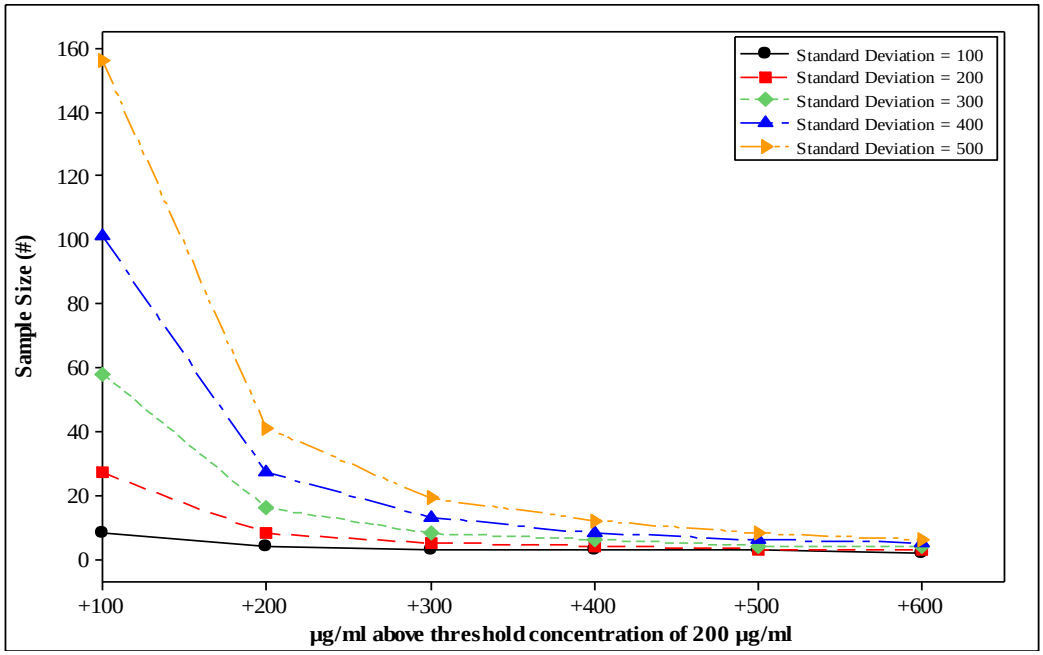


Figure 5a Sample size required to demonstrate average herd urine DHP concentrations of 3,4-DHP>200 µg/mL at a range of standard deviations.

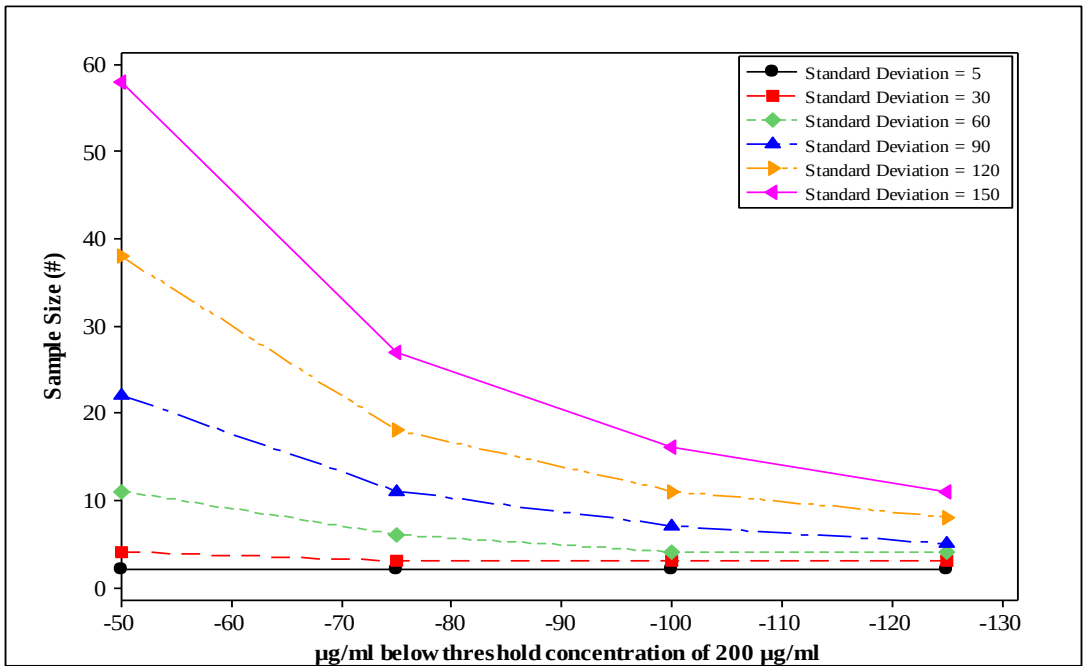


Figure 5b Sample size required to demonstrate average herd urine DHP concentrations of 2,3-DHP<200 µg/mL at a range of standard deviations.

Discussion

The results obtained in this study are in strong contrast to the findings of the original research on the efficacy of inoculation with *S. jonesii*. In that seminal work in northern Australia >25 years ago, there was rapid and almost total degradation of both DHP isomers following inoculation even in animals consuming diets high in leucaena, as indicated by very low concentrations of DHP in urine (≤50 µg/mL) (Jones and Megarrity, 1986; Hammond *et al.,* 1989b). These early studies showed that DHP levels were almost completely degraded within a few days of inoculation (Jones and Lowry, 1984; Jones *et al.,* 1985b). Jones (1994) reported that inoculation of goats in Indonesia resulted in "DHP levels declining to virtually zero after 5 days". Other work has shown that animals inoculated with DHP degrading bacteria are able to effectively degrade DHP isomers within 2-4 weeks of animals' first grazing leucaena (Pratchett *et al.,* 1991). Indeed, multiple urine sampling of a cattle herd inoculated with the original rumen fluid inoculum in 1986 indicates that the animals have retained complete protection (Peter Larsen, personal communication). These early workers also demonstrated the longevity of *S jonesii* in the rumen even with periods off leucaena of up to 9 months (Jones *et al.,* 1985b). A recent study of a closed breeding cattle herd at the CSIRO research property at Lansdown, inoculated >25 years ago using rumen fluid as the inoculum, showed that cattle have retained protection against leucaena toxicity (Jones *et al.,* 2009).

Our findings differed from this early work in several significant ways, namely: (a) there was a slow, rather than rapid, 'build up' of DHP degradation capacity over 8-12 weeks regardless of inoculation history; (b) urine frequently had high levels of 2,3-DHP, an isomer thought to be transitory only; (c) inoculation with the *in vitro* inoculum did not result in a reduction in urinary DHP on one of the 2 properties tested; and (d) 2 of 5 herds lost their protective status after only 80-111 days off leucaena pastures.

Percentage leucaena in diet and urine sampling protocol

The percentages of leucaena in the diet were considered adequate (mean 26-74%) to facilitate the intake of sufficient quantities of mimosine to induce DHP toxicity in unprotected animals (Lowry *et al*., 1983; Jones, 1994), except for properties #4 and #7 at S2. Similar diet selection levels of 30-60% leucaena have been reported in cattle herds grazing leucaena pastures in southeast Queensland during the summer/autumn period (Jones *et al.,* 1979; Jones and Jones, 1984; Galgal, 2002; Streeter, 2005).

The study employed a urine sampling technique which requires animals to be grazing leucaena immediately prior to sampling and emphasises immediate sampling of cattle upon being yarded. O'Reagain (2008) observed that mimosine and 3,4-DHP first appeared in urine 9 hours after animals first consumed leucaena, and that 3,4-DHP concentrations declined rapidly within 24 hours to ≤50 µg/mL after animals stopped consuming a 60% leucaena ration. Nevertheless there was great variability in urinary DHP concentrations within herds at all samplings. This is most likely influenced by a range of factors, namely: (a) the percentage of leucaena in diet, (b) hydration state of the animal; (c) the time between leucaena consumption and sampling; (d) the number and efficiency of DHP degrading bacteria in the rumen; and (e) rate of metabolism. All these factors may vary greatly among animals within the same herd.

There was no relationship between % leucaena in diet and urinary DHP levels. This contrasted the findings of Ghosh *et al.* (2007) who reported data from controlled feeding trials where total urine volume was collected which showed a close association between intake of leucaena (and therefore

mimosine) and urinary concentrations of DHP. However, in the latter study, there was no evidence that DHP degrading bacteria were present.

Protection status of herds prior to inoculation and success of inoculation

Our data showed high urinary DHP excretion levels at the initial sampling which indicated that 5 of the 7 herds sampled were at risk of subclinical toxicity (DHP >100 µg/mL). These herds had been grazing leucaena for between 17 – 71 days. Accordingly, at that time we concluded that herds on at least 5 properties were not protected by *S. jonesii*. However, quite unexpectedly, only 2 of the properties continued to show high excretion of DHP at S2 just prior to inoculation, indicating that degradation was occurring but had taken periods up to 72-119 days to become effective. During this time, only two properties (#1 and #7) showed signs of hair loss (switch of tail), but animals recovered rapidly with no further signs of toxicity observed. Results may have been compromised on property #8 by the early removal (>12 hrs prior to urine sampling) of animals from leucaena pasture the evening before sample collection at S2. The bulk of 3,4-DHP is cleared from the body within 24 hours following cessation of leucaena feeding (Ghosh *et al.,* 2007; O'Reagain, 2008).

The low urinary DHP concentrations detected on all properties, except #2 and #3, at the second sampling meant that the effectiveness of the *in vitro S. jonesii* inoculum (via reduced excretion of DHP compounds) could only be tested on these two properties. Following inoculation, animals on property #3 excreted DHP isomers at concentrations significantly below the threshold at S3 demonstrating that *S. jonesii* was effective. Conversely, on property #2 high levels of 3,4-DHP continued to be excreted indicating that the inoculation was not effective. This was a clear indication that inoculation carried out rigorously using recommended practice might not result in successful establishment of *S. jonesii* in the rumen.

Detection of S. jonesii using PCR

The nested PCR analysis was able to detect the presence of *S. jonesii* in only 6% of the samples. Where *S jonesii* was present (e.g. as inferred from low urinary DHP), it presumably was in populations below the sensitivity of this method ($<10^4$-10^5 cells/mL). There appeared to be a low but detectable population of *S. jonesii* on 4 properties (#1, #4, #5 and #6) prior to inoculation, which required 10-17 weeks to become effective in degrading the DHP isomers. It is known that the rumen environment can affect PCR detection sensitivity e.g. levels of carbohydrates, tannins and phenolics can interfere with DNA amplification. This might explain why some animals tested positive for *S. jonesii* on some occasions but tested negative on subsequent occasions. There are no published data, reporting abundance of *S. jonesii* measured in field–collected rumen fluid, with which to compare the results observed in this trial.

Presence of 2,3-DHP in urine samples

The detection of the compound 2,3-DHP in the urine of all herds prior to inoculation, including those herds previously inoculated, contrasts with the findings of Jones *et al*. (2009) but is consistent with other data (Dalzell *et al.,* 2012). Of the 8 herds, 4 were excreting high levels of 2,3-DHP at S1 (>100 µg/mL). The presence of this isomer, particularly on property #5, where cattle had been grazing leucaena for 10 weeks before S1, indicated that it may not be a transitory breakdown product of *S. jonesii* as previously reported (Ford *et al.,* 1984; Jones *et al.,* 2009). At the first sampling on property #5, mean 2,3-DHP concentrations were >1000 µg/mL, with concentrations ranging from non-detectable to 4408 µg/mL. These results are in accord with those of Ghosh *et al.,* (2007) who found that uninoculated animals excreted toxin concentrations which peaked at 45 ± 2.1 µg/mL mimosine, 979 ± 44.0 µg/mL 3,4-DHP and 2045 ± 192 µg/mL 2,3-DHP.

In the earlier study of Queensland cattle herds, Dalzell *et al.* (2012) reported that 32% of the 44 herds tested excreted high levels of 2,3-DHP (>200 µg/mL). Although these were 'one off' spot tests, they were conducted on herds that had been grazing leucaena for long periods and had not been recently inoculated with *S. jonesii*. Recent studies of goat herds in Thailand on long-term 100% leucaena diets also showed very high levels of 2,3-DHP in urine (>1000 µg/mL) (Phaikiew *et al.,* 2012).

The factors contributing to the accumulation of 2,3-DHP remain unclear. There are other known 2,3-DHP degrading bacteria, other than *S. jonesii*, which colonise the rumen (Hammond *et al.,* 1989b; Allison *et al.,* 1990; Dominguez-Bello and Stewart, 1991), however the presence of these bacteria in the rumen of Australian cattle has not been explored.

The efficiency and capability of DHP degradation is highly variable even between phylogenetically identical *S. jonesii* strains and is likely to be influenced by environmental conditions. For example, when *in vitro S. jonesii* cultures were deprived of 2,3-DHP for periods of ≥2 months there was a temporary or permanent loss in the 2,3-DHP degradation activity (Dominguez-Bello *et al.,* 1997; Rincon *et al.,* 2000). In another study, one in four *in vitro* cultures of rumen fluid containing *S. jonesii* could not degrade 2,3-DHP after 6 days under anaerobic storage conditions (Jones *et al.,*1985a). Therefore, the accumulation of 2,3-DHP observed in this study may be due to (a) the natural occurrence of both isomers of DHP when mimosine is degraded by plant enzymes and non-specific rumen microbes; (b) change in ability to degrade 2,3-DHP by *S. jonesii* strains; or (c) environmental conditions of the rumen limiting efficiency of DHP degradation.

Further studies investigating the toxicity implications of the presence of 2,3-DHP in ruminants and the capacity of rumen microbes to degrade both DHP isomers *in vivo* are required to understand degradation pathways and to determine how 2,3-DHP toxicity impacts animal production.

It is generally considered that both isomers are harmful. Infusion of 2,3-DHP into the rumen of sheep had an immediate deleterious impact on intake (McSweeney *et al*., 1984) and can be fatal (Puchala *et al*., 1995). Other studies have shown that high levels of 2,3-DHP in serum can reduce milk production in dairy cows (Ghosh *et al*., 2007).

DHP in blood

The herds on all properties generally had very low toxin levels in blood confirming findings from other work (Hegarty *et al.,* 1964b; Reis *et al.,* 1975; Lowry *et al.,* 1985; Smuts *et al.,* 1995; Leblanc, 2004) that DHP is quickly eliminated from blood to accumulate in the urine. Blood toxin levels were much lower than those reported by Ghosh *et al.*(2007) (155-12,982 µg/mL for 3,4-DHP and 70-2716 µg/mL for 2,3-DHP), and by Gupta and Atreja (1999) who reported 3,4-DHP levels that ranged from 97 µg/mL to 402 µg/mL. The reasons for this may relate to the method of measurement and analysis or to the capacity of animals in the present study to eliminate the toxins via the kidneys more quickly. This capacity is reported to be regulated by the form of the toxin. DHP chelated with metal ions (D'Mello and Acamovic, 1982; Puchala *et al.,* 1995) or conjugated with sugar molecules (Hegarty *et al.*, 1964a; Elliot *et al.*, 1985) may be voided more readily from the bloodstream.

Duration and diet off leucaena pasture

Two herds (properties #1 and #8) excreted large amounts of DHP when returned to leucaena pastures after 80-120 days off leucaena, which demonstrated that herds may have lost 'protection' against toxicity. This finding confirms the observations made by graziers that cattle can perform

poorly until re-inoculated when returned to leucaena after periods of leucaena-free grazing. Interestingly, the herds that grazed alternative pastures for the longest duration retained DHP degrading capability when reintroduced to leucaena pastures e.g. cattle on property #4 (grazing predominantly native blue grass pasture for 120 days) and both herds on property #5 (grazing either introduced grasses or oats/grass mixture for 112 days). Previous studies of Jones *et al.* (1985b) and Hammond *et al.* (1989a) reported that *S. jonesii* retained DHP degrading ability *in vivo* following 6-9 months without leucaena feeding although populations of *S. jonesii* appeared to decline with the length of time off leucaena.

Whilst the duration off leucaena might be an important contributor to maintenance of capacity to degrade DHP, the physical and chemical properties of the alternative diets may influence the persistence or efficacy of *S. jonesii*. The two herds that lost degradation capability grazed fresh oats or oats/grass as the alternative pasture. However, the herd grazing an oats/grass mixture on property #5 retained degradation capabilities indicating that any particular effect of oats-based diets on the persistence of *S. jonesii* in the rumen may be variable.

Nevertheless, sudden dietary changes can dramatically change rumen pH level resulting in an imbalance of microbial populations and fermentation (Eadie and Mann, 1970). *S. jonesii* populations demonstrate optimal DHP degradation and performance at a pH range of 6.0-6.8; at lower and higher pH levels DHP degradation activity is inhibited (Allison *et al.,* 1990). Highly degradable diets, such as lush actively growing forages with a high leaf to stem ratio, can lower the pH of the rumen and thus increase the risk of ruminal acidosis (Calsamiglia *et al.,* 2008). Alternatively, animals grazing forage oat pastures can experience a rumen pH level >7 (Arelovich *et al.,* 2003).

Unfortunately, as previously described, the nested PCR could not consistently detect *S. jonesii* DNA even when DHP degradation was occurring (as inferred from lop urinary DHP) and thus it was not possible to assess the rate of rundown of rumen *S. jonesii* population numbers when leucaena was removed from the diet. When the 5 herds were returned to leucaena grazing, the 3 weeks on leucaena should have been sufficient for residual populations of *S. jonesii* to increase in number in the rumen. However, *S. jonesii* was not detected despite three herds demonstrating DHP degrading capabilities. Molecular techniques which are more sensitive (by 100-1000 times) are required to monitor ruminal populations of *S. jonesii* at the relatively low levels that appear to be typically present in rumen fluid.

Variability in urinary toxin concentrations

Given the very high variability of urinary toxin concentrations among animals within a herd, a strategy for collection of an appropriate number of urine samples is required in order to statistically detect differences relative to the toxicity threshold of 100 µg/mL DHP. A strong relationship was observed between the mean toxin concentration and standard deviation i.e. standard deviations increased with mean toxin concentration (Figure 3). Thus the sample size required to accurately determine the toxicity status of herds is influenced by the degree to which mean toxin concentration exceeds, or differs from the threshold of 100 µg/mL (Figure 5). A larger sample size is required to demonstrate a significant difference from the threshold when mean toxin concentrations are close to 100 µg/mL. For example with a SD of 100, >100 samples would be required to show that 150 µg/mL was significantly above the threshold. Fewer samples (7) would be required for herds excreting >200 µg/mL above the threshold (Figure 5).

This high standard deviation in unprotected herds is not generally a major issue as herds consuming high dietary percentage of leucaena normally exhibit high urinary concentrations of

DHP (>500 µg/mL) which would require only 10 samples to demonstrate a significant difference. Therefore, it is not realistic in a practical grazing situation to attempt to demonstrate significant differences from 100 µg/mL for urine sample DHP concentrations below 250 µg/mL as greater than 15 samples would be required. In practice, we recommended that ≥10 animals from a herd be sampled to determine its toxicity status.

Liveweight gain

No significant differences in liveweight could be attributed to the effect of inoculation or pre-existing *S. jonesii* populations due to changing availability and quality of dietary components across sampling times and seasons (e.g. proportion of leucaena to grass) (Streeter, 2005).

Conclusions

Our findings showed that efficacy of the *in vitro S jonesii* inoculum to degrade mimosine and DHP in ruminants consuming leucaena contrasted with previous reports of the efficacy of *S. jonesii* in several ways:

- Degradation of DHP isomers did not occur rapidly nor completely.
- There appeared to be a residual population of DHP degraders in some herds that colonised the rumen slowly and required many weeks to effectively degrade DHP. However, it is not known if these degraders were *S. jonesii* or some other species.
- The colorimetric on-farm test kit gave poor quantitative predictions of DHP concentrations compared to HPLC but was a useful indicator of the presence and approximate concentration of urinary DHP.
- High concentrations of 2,3-DHP were often present even after long periods of leucaena grazing, an isomer previously thought to be transitory. Further work is required to demonstrate the impact of high levels of 2,3-DHP on animal performance.
- Even with rigorous inoculation procedures successful inoculation may not be achieved indicating a possible problem in the supply chain.
- Herds were found to lose protection after even relatively short periods on very different diets e.g. oats.

The nested PCR analysis indicated that *S. jonesii* numbers at the beginning of the experiment were relatively low as the DNA sequence of the type strain was detected in only a small number of cattle including those directly inoculated. Improved molecular procedures will be required to detect the relatively low rumen levels of *S. jonesii* that appear to be typical.

At a practical level, graziers are concerned about the protection status of their herds, especially the long-term effectiveness of the inoculum following seasonal grazing of non-leucaena pastures. They require an efficient system of inoculation and access to testing of herd toxicity status, as reoccurrences of toxicity will have deleterious impacts on beef production and animal health. Further research is vital to ensure the continued confidence and adoption of leucaena/grass pasture systems. It is recommended that graziers (a) test their herds annually using colorimetric

urine analysis, for presence/absence of effective populations of *S. jonesii*, particularly at the start of the season when there is an abundance of lush leucaena forage available; (b) retest herds 4-6 weeks after inoculation to confirm effective inoculation; and (c) minimize time animals spend off leucaena pastures, with a preference to maintaining 'carrier' animals on leucaena year-round to promote retention of *S. jonesii*.

Acknowledgements

We express our deep appreciation to the graziers, their families and managers who generously donated their time and resources to this research: Craig Antonio, Jonathon Schmidt, Michael Machin, Grant Shelton, Patrick Nason, Luke Hopkins, Andrew Richardson and Andrew Swan. We also thank Peter Isherwood, Graham Kerven, David Appleton, Stephen Appleton and Dr. Jennifer Waanders for assistance with laboratory analyses at the University of Queensland; Dr. Olena Kravchuk (UQ) for statistical advice; Drs Rob Dixon (DEEDI) and David Coates (CSIRO) for their valuable advice, preparation and analysis (NIRS) of faecal samples; Dr. Carl Davis (CSIRO) for advice and preparation and analysis of rumen fluid samples using the nested PCR techniques; and Hilary Stuart-Williams (The Australian National University) for analysis of faecal samples using carbon isotopes. This work received financial support from Meat and Livestock Australia (MLA).

5 Success in achieving objectives

The research team completed all the project milestones and undertook all the work required under the Terms of reference. Some objectives were partially achieved.

Objective 1 Investigate the factors affecting the presence, introduction (where required), and impact of *Synergistes jonesii* in commercial herds with problematic 'status' (with respect to their protection from DHP-induced depressions in productivity).

This project objective was partially achieved as explained below:

(a) The molecular tools (PCR and real-time PCR) developed by CSIRO were not able to detect and enumerate the unexpectedly low levels of *S. jonesii* found in the rumen fluid of the cattle pre- or post-inoculation. Even additional CSIRO work which developed a nested PCR method (with an extra step of DNA amplification) failed to detect *S. jonesii* DNA in most of the rumen fluid samples collected. The real-time PCR was unable to detect *S. jonesii* in any sample.

(b) Even though the *in vitro* inoculum was "introduced" to cattle strictly following the DEEDI guidelines, the impact of *in vitro* inoculation could be studied in just two herds, which were found to be unprotected at the time of inoculation. It successfully protected one herd but failed to protect the other herd.

The development of other more sensitive molecular tools (beyond the timeframe and budget of this project) are required to facilitate further advances in our understanding of the rumen ecology of *S. jonesii*.

Objective 2 Explore the link between *S. jonesii* and liveweight production in suspected "problem" herds.

This study did not find a relationship between animal liveweight gain and urinary toxin concentrations pre- and post-inoculation with *S. jonesii* for the following reasons:

(a) Leucaena and grass dry matter availability declined as the season progressed.

(b) The physical and chemical properties of the forage 'on offer' (i.e. forage quality), declined during the study.

(c) Grazing pressure (the number of animals per unit of forage availability) increased throughout the study and there were various 'ad hoc' changes in animal numbers and grazing management on some properties.

(d) DHP toxicity is cumulative in its effect on animal health and performance. Elevated levels of DHP in the bloodstream and suppressed blood thyroxin concentrations may take 6-8 weeks to be expressed as lowered feed intake and reduced liveweight gain. Therefore an animal may have high or low liveweight gain, at the same DHP concentration in the bloodstream (and urine), depending upon the length of its exposure to DHP toxicity.

(e) Weather conditions including rainfall and temperature fluctuated widely during the trial affecting live weight gain potential e.g. live weight gains are lower in very wet weather.

Objective 3 Investigate the post-inoculation retention of *S. jonesii* in a subset of these herds under different pasture/fodder regimes.

This objective was successfully achieved.

Objective 4 Using these results and published information, provide recommendations for managing and monitoring the retention of *S. jonesii* in cattle herds.

The research team has made recommendations to graziers for the management of leucaena toxicity.

6 Impact on meat and livestock industry – Now and in five years' time

The immediate impact of the research on the beef industry has been the demonstration that inoculation of cattle with the *in vitro S. jonesii* inoculum, and its retention in herds, does not seem to be occurring as efficiently as previously reported.

These issues may be due to problems with the *in vitro* bacterial culture itself, the supply chain of delivery of the culture to graziers or, alternatively, may be due to the nature of the non-leucaena diets and their impact on rumen ecology when cattle are moved off leucaena in winter in southern Queensland.

Consequently, continuing research is required as resolution of the problem of subclinical toxicity will have an immediate impact on the beef industry. There is continuing strong research and anecdotal evidence that the problem is widespread in cattle feeding on leucaena-grass pastures.

Addressing these problems will result in more effective production and inoculation procedures and perhaps more effective strains of *S. jonesii* bacteria. There also needs to be more effective monitoring of the toxicity status of animals by producers. At present, there is an estimated 200,000 ha of leucaena pasture established in northern Australia. Optimising animal production from these pastures by preventing leucaena toxicity will safeguard and significantly increase beef production leading to the needed production efficiencies identified by McCosker *et al.* (2010). An increase of 50 kg per animal due to effective inoculation (i.e. ~20% of annual LWG of 250 kg/hd/yr), from the estimated 133,000 cattle accessing leucaena, is valued at >$11.5 million/year (at $1.75/kg liveweight).

In 5 years' time, even larger areas of leucaena will be established and therefore the production benefits from preventing leucaena toxicity will be further amplified. Further research is therefore needed to identify the most effective strains of bacterium, the ideal conditions for preserving *in vitro* sources of inoculum (freezing or lyophilisation), and the best practice for administering inoculum to animals (via direct rumen injection). This will help ensure producers can realise the potential productivity of leucaena-based pastures.

7 Conclusions and recommendations

The research program described in this report investigated the efficacy and persistence of the *in vitro* source of *S. jonesii* inoculum when introduced to cattle in commercial herds grazing leucaena in Queensland. The research was conducted on 8 commercial cattle properties in southern Queensland with established leucaena pastures.

7.1 Efficacy and persistence of *in vitro* inoculum

The efficacy of inoculation with *in vitro* inoculum could be evaluated on 2 of the 8 properties only, with inoculation eliminating toxicity on one property and failing on the other. Cattle on the other 6 properties apparently acquired DHP degrading capabilities during a period of 10-17 weeks grazing leucaena from either (1) populations of *S. jonesii* below the detection of the PCR tests, (2) residual variant populations of *S. jonesii*, present at populations below the level of detection of real-time PCR, or (3) from a population of DHP-degrading bacteria not yet identified. The development of degradation capacity was very slow compared with results from inoculation experiments using rumen fluid and published more than 20 years ago. It was expected that DHP excretion would cease within 1-2 weeks after inoculation if effective *S. jonesii* bacteria were present.

PCR results indicated that the *S. jonesii* variant had different 16S rDNA to the type strain of *S. jonesii* cultured in the *in vitro* inoculum. The type strain was not detected in rumen fluid samples from any animal after inoculation. Thus the PCR data could not be used to confirm that the oral drench inoculations were successful or that the *in vitro S. jonesii* inoculum had colonised the rumen after inoculation.

The retention study, in which inoculated cattle were moved off leucaena onto alternative non-leucaena pastures, showed that *S. jonesii* maintained the capacity to degrade DHP in cattle on two of four properties (3 of 5 herds). This loss of protection occurred more quickly than previously reported and thus supported our hypothesis that properties where cattle herds had been previously inoculated could lose protection. In this study, the time spent off leucaena was not the critical factor

as herds were off leucaena for similar periods (80-120 days). There was an indication that the loss may be diet related as herds that lost protection grazed similar pasture types i.e. lush oats pasture. We hypothesise that sudden dietary changes may contribute to changes in the function, stability and environment of the rumen which may cause a decline, or loss, of bacterial numbers or suppress the efficacy of the *S. jonesii* bacteria.

7.2 Practical applications and future research directions

Despite the equivocal outcome of this research, it is anticipated that many commercial herds grazing leucaena pastures in Queensland, even those previously inoculated with the *in vitro* source of *S. jonesii*, may have lost 'protection' due to either failed inoculation/colonisation or to periods off leucaena. In these and other herds, where slow re-establishment of effective populations of *S jonesii* occurs, cattle will be experiencing undiagnosed subclinical toxicity that may limit their productivity and profitability.

Nevertheless, despite indications of reduced efficacy, we concluded that the *in vitro* inoculum should continue to be used commercially until improved methodology is available.

Pending further research, it is recommended that graziers incorporate the following practices into their leucaena toxicity management program:

(a) Continue using the *in vitro S. jonesii* inoculum until improved inoculation methodology and application procedures are developed;

(b) Regularly (at least annually) test herd toxicity status particularly at the start of the growing season when there is an abundance of lush leucaena forage. Cattle herds should be grazed on leucaena for a minimum of 3-4 weeks to promote high leucaena intake prior to toxicity testing using the University of Queensland colorimetric urine test;

(c) Retest herd toxicity status 4-6 weeks after inoculation to confirm successful inoculation and colonisation of the rumen by *S. jonesii*;

(d) Minimise time animals spend off leucaena pastures with a preference for maintaining 'carrier' animals on leucaena year-round to promote retention and capacity of *S. jonesii* to degrade DHP isomers

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9 Appendices

9.1 Appendix 1

MLA Terms of Reference for the research program - sent by email on 4/04/2007

Terms of Reference

MLA Northern Beef Program

Investigations into leucaena-induced toxicity in beef cattle

The Meat and Livestock Australia Northern Beef Program is seeking a research team or teams to:

1. Establish the *in vivo* effectiveness of the current source of *Synergistes jonesii* for degrading 3,4-DHP and 2,3-DHP, including the development of a real-time PCR test (or similar) able to distinguish the presence and enumerate strains of *Synergistes jonesii* in rumen fluid. Provide recommendations for ensuring the ready availability of effective sources of *Synergistes jonesii* for dosing cattle grazing leucaena.
2. Identify up to 8 properties (in conjunction with MLA) that are potentially experiencing subclinical leucaena toxicity and:
    - Document herd history and monitor grass:leucaena intake, urinary DHP levels, rumen *Synergistes jonesii* levels and liveweight gain performance
    - Where appropriate, dose herds with *Synergistes jonesii* and monitor subsequent urinary DHP levels, rumen *Synergistes jonesii* levels and liveweight gain performance
    - Using these results and published information, provide recommendations for ensuring the successful introduction and retention of *Synergistes jonesii* in cattle herds

Collaborative and integrated proposals that address both outcomes are preferred, but proposals that address one or other of the required outcomes will be considered.

Selection Criteria and Process:

The project proposal should be submitted in the standard MLA full proposal format (templates can be downloaded from mla.com.au) to address these Terms of Reference.

Selection of the successful proposal will be based on the following criteria:

- Ability to achieve the project objectives;
- Soundness of the method proposed, including timeframes and milestones by which progress will be measured;
- Experience of the nominated researcher and proposed team members; and
- The quoted price for the work (including overall fees, the proposed timeframe, number of days and the daily fee rate).

Project Proposal Submissions:

Proposals may be lodged by post or electronically (preferred) to:

Rodd Dyer

Northern Beef Program Manager

Meat & Livestock Australia

PO Box 2363

Newstead QLD 4006

Phone: 07 3620 5234

Fax: 07 5464 2898

Mobile: 0429 486 902

Email: rdyer@mla.com.au

Proposals must be received by **COB 18th May 2007**

9.2 Appendix 2

UQ Animal Ethics approval certificate for this research program

THE UNIVERSITY OF QUEENSLAND

PLEASE KEEP THIS FORM IT IS YOUR RECORD OF YOUR AEC APPROVAL NUMBER

Ms Ann Higgins
Animal Welfare Coordinator
Research and Research Training Division
Cumbrae Stewart Building (72)
St Lucia Q 4072
Ph: (07) 3365 2713 Fax: (07) 3365 4455
Email: a.higgins@research.uq.edu.au

ANIMAL ETHICS APPROVAL CERTIFICATE Date: 18-Dec-2008

Dear Dr Max Shelton, Land, Crop and Food Sciences

The following project: *Presence, impact and retention of rumen bacterium Synergistes jonesii in beef cattle grazing leucaena*

Requesting funding from (Grant Awarding Body):- MLA involves animal experimentation. It has been reviewed and ethical clearance obtained from the University Animal Ethics Committee (Production and Companion Animal).

AEC Approval Number: SLAFS/SAS/944/08/MLA

Previous AEC Number:

Approval Duration: 05-Jan-2009 to 05-Jan-2011

Permit(s):

SUBSPECIES	STRAIN	CLASS	GENDER	SOURCE	AMOUNT
Cattle		Other	Mix	Privately owned	320

Proviso(s):

Please use this Approval Number:
1. When ordering animals from Animal Breeding Houses
2. For labelling of all animal cages or holding areas. In addition please include on the label, Chief Investigator's name and contact phone number.
3. When you need to communicate with this office about the project.

It is a condition of this approval that all animal usage details be made available to Animal House OIC. (UAEC Ruling 14/12/2001)

This certificate supercedes all preceeding certificates for this project (i.e. those certificates dated before 18-Dec-2008

9.3 Appendix 3

Synopsis of the research project sent to potential grazier collaborators

UQ/MLA Project

Presence, impact and retention of *Synergistes jonesii* in 'problem' herds grazing leucaena

Animal Ethics Approval

AEC Approval Number: SLAFS/SAS/944/08/MLA

Purpose and description

There are suggestions that the current DEEDI *in vitro* source of rumen inoculum containing *Synergistes jonesii* is not working effectively to fully degrade harmful mimosine by-products, 3,4-DHP and 2,3-DHP, produced during leucaena digestion in cattle. The reasons for this may relate to factors affecting the introduction, spread and retention of *S. jonesii* within a mob or to reduced effectiveness of bacterial strains from the current inoculum cultures. The latter possibility has been explored in other work.

This project will explore the factors affecting the presence and dynamics (introduction, impact and retention) of *S. jonesii* in mobs of cattle grazing leucaena suspected to have lost protection from the bacteria on commercial enterprises. It will also explore whether sub-clinical impacts of mimosine toxicity in these herds is adversely affecting liveweight production.

Objectives

The project aims to:

1. Investigate the factors affecting the presence, introduction (where required), and impact of *Synergistes jonesii* in commercial herds with problematic 'status' (with respect to their protection from DHP-induced depressions in productivity).

2 Explore the link between *S. jonesii* and liveweight production in suspected "problem" herds.

3. Investigate the post-inoculation retention of *S. jonesii* in a subset of these herds under different pasture/fodder regimes.

4. Using these results and published information, provide recommendations for managing and monitoring the retention of *S. jonesii* in cattle herds.

Methodology

The project is comprised of 2 experiments. The first experiment will study the impact of DHP toxicity on liveweight gain. The second will investigate changes in *S. jonesii* populations in the rumen of cattle once leucaena is removed from the diet and animals are fed other forages/rations.

Experiment 1

This experiment aims to observe the impact leucaena toxicity has on the liveweight gain of unprotected cattle grazing leucaena.

Outline

Unprotected animals are required to graze leucaena for 4-6 weeks to develop subclinical toxicity. Cattle will then be inoculated with *S. jonesii* rumen bacteria (sourced from DEEDI) in accordance with the industry's current recommended protocol. The animal liveweight performance, *S. jonesii* populations in rumen fluid, leucaena intake and DHP levels (urine and blood) will be monitored.

Activity Schedule

1) Preconditioning period:

Animals are required to graze leucaena for >3 weeks prior to our first visit and sample collection to ensure sufficient levels of toxins have been consumed by cattle to induce toxicity. This period will also ensure adequate time for *S. jonesii* populations to recolonise the rumen if present in your animals.

2) First sample collection:

A subset of 25 animals will be selected from your herd for use in the trial. They will be tagged and weighed. Samples of blood (tail bleeding), rumen fluid, urine and faeces will be collected from a subset of 10-15 of these animals. These samples will determine the protection status of your herd before commencing the trial. In particular, urine will be used to determine the level of DHP present, while the rumen fluid will be analysed to determine the presence or absence of *S. jonesii* and numbers of bacteria present. If any animal tested has effective *S. jonesii* present the herd cannot be used in the trial.

3) Second sample collection:

The 25 selected animals will be weighed again 4-6 weeks after the first sample. Blood, urine, faeces and rumen fluid samples will be collected from the same subset of 10-15 animals. This is a precautionary measure to ensure animals still remain ‘bug’ free. These animals will then be inoculated with inoculum sourced from the DEEDI following current industry best practice as follows - 10% of the herd will be orally drenched with 100 mL of *in vitro* inoculum.

4) Third sample collection:

All 25 animals will be weighed and the same subset animals (10-15) will be sampled another 4-6 weeks after the second sample. This period following inoculation with *S. jonesii* should

be sufficient to allow the 'bug' to populate the rumen of inoculated animals. It should also be long enough for the bacterium to spread to other animals in the herd.

Procedures

Blood collection:

Approximately 10 mL of blood will be collected from each animal using the tail bleeding method.

Rumen fluid collection:

This involved the insertion of a small pipe down the mouth of the animal into the rumen. The collection of 10-50 mL is required.

Urine and faeces collection:

Urine samples will be collected when animals voluntarily urinate. Faecal samples will be collected by the rectal grab method.

Experiment 2

Outline

Cattle used in Experiment 1 will be removed from leucaena pastures and then fed commercial leucaena-free diets of oats, grass plus urea supplementation and/or a grain based ration. Populations of *S. jonesii* will be monitored over a 4-6 month period after leucaena feeding ceases.

Activity Schedule

Animals studied in 3-5 herds in Experiment 1 will be removed from leucaena pastures and placed on an alternative diet to monitor the effects this change has on the rumen populations of *S. jonesii*. The diets involved will depend largely on what is typically used in the enterprise. The subset of animals will be split into 2 groups and fed different diets, and monitored. Over a 6 month period 6 rumen fluid samples will be taken to monitor the *S. jonesii* population dynamics. Frequency of sampling will be more intense following the removal of leucaena from the diet (e.g. fortnightly) and then extend to monthly intervals.

Questions for Graziers

What area is currently planted to leucaena? What resources would be available?

UQ needs to know the inoculation history of property/herd. Have you ever deliberately introduced *S. jonesii* by: A) acquiring and administering inoculum; or B) mixing animals thought to be protected by *S. jonesii* to acquire the bacterium by animal-to-animal transfer?

Inducing leucaena toxicity could result in reduced liveweight gains during the 7-9 week period cattle are not protected. Animals should recover quickly after inoculation and compensate for these losses. Are you prepared to suffer production losses during the research program?

For successful implementation of Experiment 1, all animals must have sufficient leucaena available at all times. Would you be willing to lower the stocking rate of your paddock(s) to ensure adequate leucaena supply for the duration of the project?

Would inducing toxicity and then collecting urine, blood, faeces and rumen fluid from your animals raise animal welfare concerns for you?

The project requires the study of herds suffering from subclinical DHP toxicity. Does the grazier have doubts regarding the protection status of their herd?

Does the property have a good set of cattle yards and a veterinary crush with a kick gate for the safe collection of blood, urine, faeces and rumen fluid samples?

Accurate scales are required for regular cattle weighing. Are they available?

Would you be interested in participating in the retention trial (Experiment 2)? What post-leucaena diet options (oats, pasture/urea supp, feedlot) exist on the property?

Sample collection needs to occur every 4-6 weeks, this can be flexible give or take a week, but for research purposes any longer will not be suitable. Would this be a problem for you regarding labour demands for mustering and handling animals?

9.4 Appendix 4

Consent form signed by collaborating graziers

Grazier Consent Form

Project title:	Presence, impact and retention of *Synergistes jonesii* in 'problem' herds grazing leucaena
Funding agency:	Northern Beef Program, Meat Livestock Australia, B.NBP.0494
Research organization:	The University of Queensland
UQ AEC Approval No:	SLAFS/SAS/944/08/MLA

Objectives of research program

The browse legume leucaena (*Leucaena leucocephala*) can be toxic to grazing cattle. The toxic agent is the amino acid mimosine and its rumen breakdown product DHP. Although rarely fatal, hidden (subclinical) DHP toxicity can reduce animal productivity. Inoculating cattle with the rumen bacterium *Synergistes jonesii*, should prevent leucaena toxicity.

There are suggestions that the current *in vitro* source of rumen inoculum containing *S. jonesii* is not working effectively to fully degrade harmful mimosine by-products, 3,4-DHP and 2,3-DHP, produced during leucaena digestion in cattle. The reasons for this may relate to factors affecting the introduction, spread and retention of *S. jonesii* within a mob or to reduced effectiveness of bacterial strains from the current inoculum cultures. The latter possibility has been explored in other work. This project will explore the factors affecting the presence and dynamics (introduction, impact and retention) of *S. jonesii* in mobs of cattle grazing leucaena suspected to have lost protection from the bacteria on commercial enterprises. It will also explore whether sub-clinical impacts of mimosine toxicity in these herds is adversely affecting liveweight production.

Unprotected cattle will graze leucaena pastures before and after inoculation with *S. jonesii* and liveweight gain will be measured. Paired samples of blood, urine, faeces and rumen fluid will be collected at least 3 times to measure toxin levels (urine/blood), the proportion of leucaena in diet (faeces) and the rate of spread and changes in *S. jonesii* populations in animals in the herd following inoculation (rumen fluid).

I ..... Owner/Manager of the property ..... located at ....

give Sam Graham, Scott Dalzell, Max Shelton and their colleagues permission to enter my property and collect urine, blood, rumen fluid and faecal samples from 10-15 of my cattle for research purposes.

Signature: .....

Date: .....

9.5 Appendix 5

Examples of Nested and Real-time PCR primers, standard curves and detection limits for *S. jonesii*

Nested PCR primers and detection limits

First round PCR, 60F-CAT/1275 R

Nested PCR, 998F/1091R

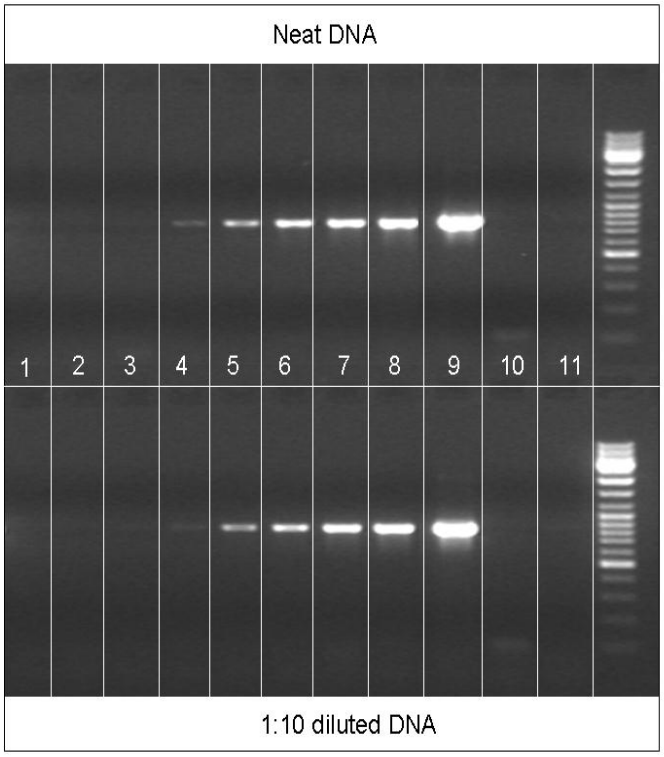

Figure 1 PCR products amplified from nested PCR primers 60F-CAT/1275 R (initial amplification) and 998F/1091R (nested amplification). Lane

1. Rumen sample – *S. jonesii* (nil cells)
2. Rumen sample + *S. jonesii* (10^2 cells)
3. Rumen sample + *S. jonesii* (10^3 cells)
4. Rumen sample + *S. jonesii* (10^4 cells)
5. Rumen sample + *S. jonesii* (10^5 cells)
6. Rumen sample + *S. jonesii* (10^6 cells)
7. Rumen sample + *S. jonesii* (10^7 cells)

8. Rumen sample + *S. jonesii* (10^8 cells)

9. Positive control: pure *S. jonesii* genomic DNA

10. Negative DNA control: pure Synergistes str. MFA1 genomic DNA

11. Negative control: No template control

Molecular weight marker: 1 kb plus Generuler (Fermentas)

Real-time primers - standard curves

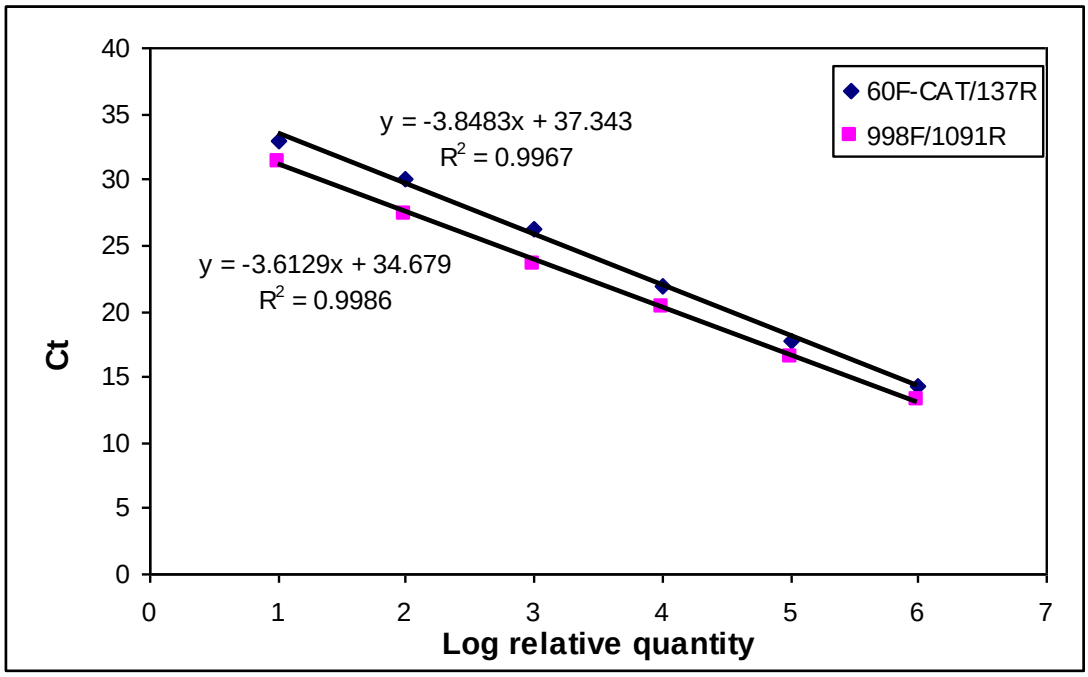

Figure 2 Standard curves for real-time primers 60F-CAT/137R and 998F/ 1091R constructed using genomic DNA from a pure culture of *S. jonesii.* Note: This represents detection of actual target genes for *S. jonesii* in the PCR reaction.

Real-time primers - Standard curves over detectable limits

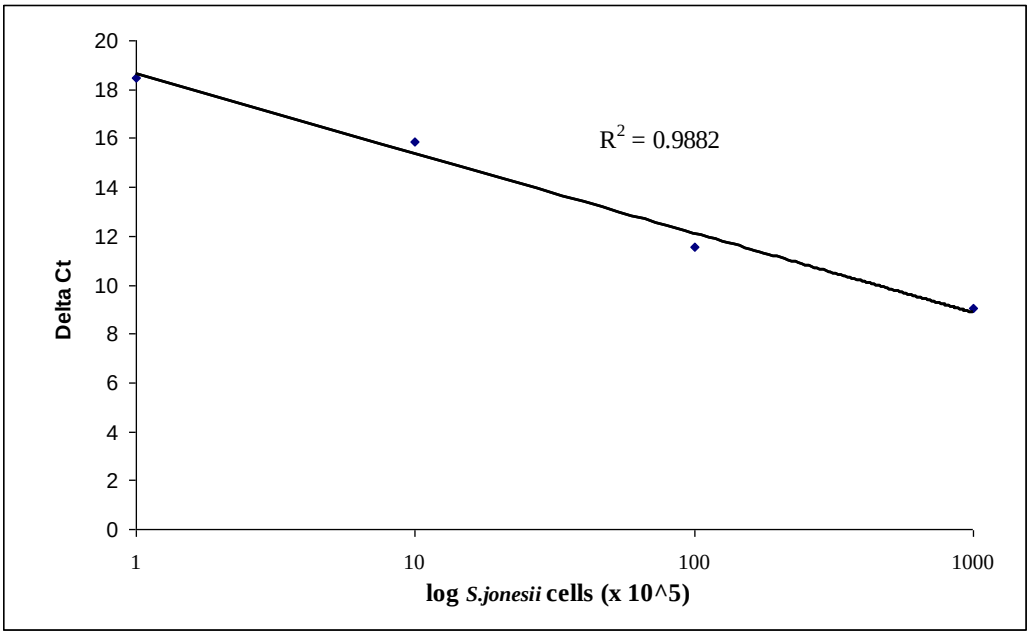

Figure 3 Standard curve for real-time primers 998F/1091R using a rumen sample containing known quantities of *S. jonesii* (10^5 to 10^8 cells/mL) normalised relative to the total quantifiable bacterial population. Note: This represents detection limits after rumen sample extraction and subsample used for PCR reaction.

9.6 Appendix 6

Collaborating Graziers in The University of Queensland Leucaena Toxicity Study

Property 1

Location: Millmerran

Inoculation history: New leucaena grower - never inoculated cattle

Cattle: 150 weaner Angus steers and heifers (250 kg LW) grazed 150 acres of leucaena.

Sample dates: Cattle started grazing leucaena - 23/2/09

Sample 1 - 3/4/09 (after 45 days grazing leucaena to determine toxicity status)

Sample 2 - 15/5/09 (after 87 days grazing leucaena before inoculation)

Sample 3 - 26/6/09 (after 42 days grazing leucaena following inoculation)

Trial 1 - The impact of leucaena toxicity on animal health & production

The impact of leucaena toxicity on animal health and production was studied in cattle grazing leucaena before and after inoculation with DEEDI *Synergistes jonesii* inoculum following the current industry recommended protocol.

KEY FINDINGS

1. The cattle were protected from leucaena toxicity at the end of Trial 1.
2. The cattle consumed significant amounts (>30%) of leucaena in their diets throughout the trial, enough to induce toxicity in unprotected animals.
3. Cattle excreted high levels of 3,4-DHP in their urine indicating they were suffering leucaena toxicity during the 45 day period ending at Sample 1. Surprisingly, by Sample 2 the cattle appeared to have gained protection (that is they acquired DHP degrading rumen micro-organisms) before UQ inoculated the herd with DEEDI inoculum, as evidenced by low levels of both 3,4- and 2,3-DHP in the urine.
4. It is possible that as these cattle were bought from the saleyards they may have had previous exposure to cattle inoculated with *S. jonesii*. Alternatively, other cattle (e.g. bulls) brought onto *the property* might have been exposed to *S. jonesii* and thereby introduced the bacteria into the environment from which it colonized the trial cattle. It is also possible, although unlikely, that *S. jonesii* bacteria survived the cleaning protocol used to sterilize the rumen pumping equipment and that the research team inadvertently transferred viable bacteria to the herd at Sample 1.

5. Very low numbers of *S. jonesi*i were found in rumen fluid (RF). DNA tools developed to determine the presence/absence of *S. jonesii* and to count their numbers in RF were not sensitive enough to enable changes in bug populations in the rumen of individual animals or the rate of spread between animals to be monitored.

6. *Synergistes jonesii* bacteria were detected in only 1 animal (pink tag#18 or NLIS #524) at Sample 3 and were genetically different to the DEEDI inoculum used.

7. Bacteria with the same genetic code as the DEEDI inoculum were not detected in RF at Sample 3.

8. There were no obvious trends in cattle liveweight gain related to toxicity.

Trial 2 - The retention of *S. jonesii* in cattle removed from leucaena

The cattle from Trial 1 were removed from the leucaena pasture immediately following Sample 3 and were placed on an oats pasture for 111 days. The ability of *S. jonesii* to persist in the rumen was studied by placing the cattle back on leucaena for 21 days before checking them for signs of toxicity.

KEY FINDINGS

1. Cattle lost viable populations of *S. jonesii* after grazing oats for 111 days in Trial 2 and were <u>not protected</u> from leucaena toxicity.

2. The rate of decline in *S. jonesii* populations could not be monitored because they exist in low populations in the rumen, below the level of sensitivity of current DNA tools of measurement.

MANAGEMENT RECOMMENDATIONS

1. Inoculate cattle grazing leucaena with DEEDI *S. jonesii* bacteria. Follow the current recommended protocol by inoculating 10% of the herd, 2 weeks after they start consuming leucaena.

2. Maintain 'carrier' animals on leucaena pastures throughout winter to ensure *S. jonesii* is maintained in their rumen and can then spread throughout a new herd when introduced to leucaena the following December/January.

3. Routinely check the toxicity status of 'carrier' and production animals annually using the urine test kit to ensure your herd is protected.

MORE DETAILED ANALYSIS

Trial 1 - The impact of leucaena toxicity on animal health & production

Indicators of toxicity were measured in 15 head before and after inoculation with *S. jonesii*.

Amount of leucaena in diet

The proportion of leucaena in the diet selected by the cattle was estimated by analysing faeces samples using the delta carbon method. The average amount of leucaena in the diet of the cattle varied from 66% at the start of the experiment to 32% at the end of the study period. At these levels of leucaena intake we would expect unprotected animals to excrete significant quantities of the DHP toxins. It is also important to note that cattle only need 35-40% leucaena in diet to achieve 1 kg/hd/d liveweight gain.

Toxicity status of the herd

Urine

The concentration of the toxins 3,4-DHP and 2,3-DHP was measured in the urine samples by high performance liquid chromatography. Unprotected cattle grazing leucaena excrete these toxins in concentrations exceeding 200 ppm (or mg/l). Cattle excreted high levels of 3,4-DHP at Sample 1 (average = 755 ppm), with 14 of the 15 animals exceeding the toxicity threshold of 200 ppm. At this time cattle excreted very little 2,3-DHP indicating that *S. jonesii* was not present in the rumen of these animals. Surprisingly, all cattle were excreting little 3,4-DHP at Sample 2 (87 days after they started grazing leucaena) prior to inoculation with the DEEDI inoculum. It would appear that the cattle somehow picked up rumen microbes capable of degrading the toxin. After inoculation of the cattle with DEEDI inoculum, animals continued to excrete low concentrations of both toxins as was expected. However, 1 animal at Sample 3 did have 3,4-DHP present in its urine at 208 ppm.

Blood

Very low concentrations of the toxins (<20 ppm) were detected in the blood of the animals throughout the experiment. There was no relationship between blood and urine DHP concentrations. These findings indicate that measuring blood toxin concentrations will be of little use in determining the toxicity status of animals.

Rumen fluid

Two DNA tests were conducted on the rumen fluid (RF) samples. The first method tried to detect whether *S. jonesii* DNA was present in the RF. The second method tried to count the numbers of *S. jonesii* bacteria present in the RF. The trial found that *S. jonesii* was present in very low numbers ($<10^5$ bacteria/ml) in the RF of cattle grazing leucaena pastures compared to other common rumen bacteria. Even at these low populations, *S. jonesii* appeared to prevent leucaena toxicity.

These new techniques developed to detect and count *S. jonesii* were not sensitive enough to detect any bacteria in the cattle tested. CSIRO then developed an even more sensitive DNA detection tool and it detected *S. jonesii* in only 1 animal (steer pink tag #18 or NLIS #524) at Sample 3. This animal had not received the oral drench with the DEEDI inoculum. Other more sensitive molecular techniques for detecting and counting *S. jonesii* in RF are being investigated.

It is interesting to note that the DNA of the bacteria found in animal #18 had slightly different combinations of DNA base pairs compared to those of *S. jonesii* produced in the DEEDI inoculum, indicating that the *S. jonesii* detected was a genetically different strain to the *S. jonesii* in the original *in vitro* inoculum used. Possible explanations for this genetic change to *S. jonesii* are: 1) the original DEEDI bacteria mutated in the rumen; or 2) the DEEDI bacteria did not persist in the rumen and the different strain of bacteria found came from another source (e.g. the environment or other carrier cattle).

Summary Table 1 Data from cattle grazing leucaena before (Samples 1 & 2) and after (Sample 3) inoculation with *S. jonesii*.

	Sample 1	Sample 2	Sample 3
Leucaena in diet (%)			
Average	66%	36%	32%
Range	60-78	24-49	14-43
Urine 3,4-DHP concentration (ppm)			
Average	775 ppm	32 ppm	45 ppm
Range	185-1917	4-108	0-211
Urine 2,3-DHP concentration (ppm)			
Average	27 ppm	2 ppm	2 ppm
Range	12-56	0-10	0-20
Liveweight gain (kg/hd/d)			
Average	0.75	0.89	-0.48
Range	0.18-1.16	0.33-1.55	-1.02 to -0.02

Note - ppm is equal to mg/l

Liveweight gain

Over the 129 days of the trial cattle gained an average of 0.39 kg/hd/d. Cattle averaged 0.75 kg/hd/d (range 0.18 to 1.16 kg/hd/d) liveweight gain over the first grazing period, 0.89 kg/hd/d (range 0.33 to 1.55 kg/hd/d) over the second and -0.48 kg/hd/d (range -1.02 to -0.02 kg/hd/d) over the third. The research team does not think that LWG was affected by leucaena toxicity during the trial due to the absence of toxin excretion in cattle at Samples 2 & 3. The poor performance over the last grazing period coincided with a shortage of feed supply (both leucaena and roughage) and a period of 2 weeks of cold wet miserable weather.

Trial 2 - The retention of *S. jonesii* in cattle removed from leucaena

Eight (8) cattle from Trial 1 were removed from the leucaena pasture immediately following Sample 3 (26/6/09) and were placed on to an oats cv. Taipan pasture. The population of *S. jonesii* was monitored in rumen fluid (RF) samples after 6, 13, 20, 27, 34, 48, 83 and 111 days as the cattle grazed oats. After 111 days (15/10/09) the cattle were returned to a leucaena pasture for 21 days before blood, urine, faeces and RF samples were taken on 5/11/09 to determine if *S. jonesii* had survived in the absence of leucaena in the cattle's diet.

Population dynamics of *S. jonesii* in the rumen

Due to the problem of not having enough sensitivity in our DNA analysis, it was not possible to monitor changes in bug populations over time grazing oats as we had hoped. *Synergistes jonesii* DNA was not detected in any of the 90 RF samples taken from the 15 animals over the 6 sample dates. We assume that the bacterium was present in the early RF samples (times 0-1 week after removing cattle from leucaena) but we have no idea how populations changed over time in the RF of cattle grazing oats.

Amount of leucaena in diet

Once the cattle had been returned to a leucaena pasture, the proportion of leucaena in diet averaged 64% (range 62-66%), see Summary Table 2 below. This indicated that the animals had very similar grazing behaviour selecting a diet with a consistent amount of leucaena in it. These high levels of leucaena intake would have enabled *S. jonesii* populations to build up in the rumen of cattle had they survived the period of absence of leucaena in diet. Over 60% leucaena in diet will also induce toxicity in animals not protected by *S. jonesii*.

Toxicity status of the herd

Urine

Urine analysis indicated that the cattle were suffering from leucaena toxicity 3 weeks after they resumed eating leucaena. Animals were excreting a mean 3,4-DHP toxin concentration of 950 ppm and all animals had urinary concentrations in excess of the toxicity threshold of 200 ppm. These levels were higher than those recorded at Sample 1 in Trial 1, even though cattle were consuming the same amount of leucaena in diet. Concentrations of 2,3-DHP were lower, however 2 animals were excreting this toxin in excess of the 200 ppm threshold.

Blood

Blood toxin concentrations were very low as observed in Trial 1 and were not a useful indicator of the toxicity status of the animals.

Summary Table 2 Data from cattle fed leucaena after 111 days grazing oats

	After 21 days back on leucaena
Leucaena in diet (%)	
Average	64%
Range	62-66
Urine 3,4-DHP concentration (ppm)	
Average	950 ppm
Range	515-1693
Urine 2,3-DHP concentration (ppm)	
Average	183 ppm
Range	73-476

Note - ppm is equal to mg/l

Property 2.

Location: Wandoan

Inoculation history: Problem herd - cattle previously inoculated but may have lost protection

Cattle: 30 Brahman × Charbray steers (370 kg LW) grazed 90 ac leucaena + 140 ac grass

Sample dates: Cattle started grazing leucaena - 20/12/08

Sample 1 - 12/2/09 (after 54 days grazing leucaena to determine toxicity status)

Sample 2 - 8/4/09 (after 110 days grazing leucaena before inoculation)

Sample 3 - 27/5/09 (after 50 days grazing leucaena following inoculation)

The impact of leucaena toxicity on animal health & production

The impact of leucaena toxicity on animal health and production was studied in cattle grazing leucaena before and after inoculation with DEEDI *Synergistes jonesii* inoculum following the current industry recommended protocol.

KEY FINDINGS

1. It was unclear if the cattle appeared to be suffering leucaena toxicity at the beginning of the trial, based upon the colorimetric test of urine samples. A second series of urine samples were collected and analysed 2 weeks after Sample 1 and stronger colour was detected. HPLC indicated that only 1 animal had urinary 3,4-DHP exceeding 200 ppm at Sample 1. Unfortunately, the second series of samples were not analysed by HPLC.

2. The cattle were considered protected from leucaena toxicity at the end of the trial, although some animals had high levels of 2,3-DHP in their urine.

3. The cattle consumed high levels (36-74%) of leucaena in their diets throughout the trial; high enough to induce toxicity in unprotected animals.

4. Cattle excreted low levels of both 3,4-DHP & 2,3-DHP in their urine indicating they were not suffering leucaena toxicity at Sample 2. Following inoculation of the herd with DEEDI inoculum low levels of 3,4-DHP but erratic and higher levels of 2,3-DHP were observed in the urine samples at Sample 3.

5. *Synergistes jonesii* bacteria were only detected in 4 of the RF samples collected throughout the trial.

6. Bacteria with the same genetic code as the DEEDI inoculum were not detected in RF at Sample 3.

7. We assume very low numbers of *S. jonesi*i were present in the rumen fluid (RF) samples given DHP was being detoxified by the cattle. DNA tools developed to determine the presence/absence of *S. jonesii* and to count their numbers in RF were not sensitive enough to enable changes in bug populations in the rumen of individual animals or the rate of spread between animals to be monitored.

8. There were no obvious trends in cattle liveweight gain related to toxicity.

MANAGEMENT RECOMMENDATIONS

1. Inoculate cattle grazing leucaena with DEEDI *S. jonesii* bacteria. Follow the current recommended protocol by inoculating 10% of the herd, 2 weeks after they start consuming leucaena.

2. Maintain 'carrier' animals on leucaena pastures throughout winter to ensure *S. jonesii* is maintained in their rumen and can then spread throughout a new herd when introduced to leucaena the following spring/summer.

3. Routinely check the toxicity status of 'carrier' and production animals annually using the urine test kit to ensure your herd is protected.

MORE DETAILED ANALYSIS

The impact of leucaena toxicity on animal health & production

Indicators of toxicity were measured in 15 head before and after inoculation with *S. jonesii*.

Amount of leucaena in diet

The proportion of leucaena in the diet selected by the cattle was estimated by analysing faeces samples using the delta carbon method. The average amount of leucaena in the diet of the cattle ranged from 36-72% throughout the experiment. At these high levels of leucaena intake we would expect unprotected animals to excrete significant quantities of the DHP toxins. It is also important to note that cattle need 35-40% leucaena in diet to achieve 1 kg/hd/d liveweight gain.

Toxicity status of the herd

<u>Urine</u>

The concentration of the toxins 3,4-DHP and 2,3-DHP was measured in the urine samples by high performance liquid chromatography. Unprotected cattle grazing leucaena excrete these toxins in concentrations exceeding 200 ppm (or mg/l). Cattle excreted low but variable levels of 3,4-DHP (average = 27 ppm) and 2,3-DHP (average = 43 ppm) at Sample 1, with 1 of 14 and no animals exceeding the toxicity threshold of 200 ppm for 3,4-DHP and 2,3-DHP respectively. Because the colour spot test was inconclusive at Sample 1, a second batch of samples was collected 2 weeks later and exhibited stronger red colour development indicating that toxicity may have been a problem. At Sample 2 the cattle were excreting low levels of both 3,4-DHP (average = 10 ppm) and 2,3-DHP (average = 30 ppm) 110 days after they started grazing leucaena. After inoculation of the cattle with DEEDI inoculum, animals

excreted low concentrations of 3,4-DHP (average = 9 ppm) but surprisingly variable concentrations of 2,3-DHP (average = 122 ppm) with 3 animals exceeding the 200 ppm threshold..

Blood

Very low concentrations of the toxins 3,4-DHP and 2,3-DHP (<20 ppm) were detected in the blood of the animals throughout the experiment. There was no relationship between blood and urine DHP concentrations. These findings indicate that measuring blood toxin concentrations will be of little use in determining the toxicity status of animals.

Rumen fluid

Two DNA tests were conducted on the rumen fluid (RF) samples. The first method tried to detect whether *S. jonesii* DNA was present in the RF. The second method tried to count the numbers of *S. jonesii* bacteria present in the RF. The trial found that *S. jonesii* was present in very low numbers ($<10^5$ bacteria/ml) in the RF of cattle grazing leucaena pastures compared to other common rumen bacteria. Even at these low populations, *S. jonesii* appeared to prevent leucaena toxicity.

The new DNA techniques developed to detect and count *S. jonesii* were not sensitive enough to detect any bacteria in the cattle tested. CSIRO then developed an even more sensitive DNA detection tool. It could detect *S. jonesii* in only 4 of the RF samples collected: animal tag# 6 at Samples 2 & 3 and animal tag# 14 at Sample 2 and #19 at Sample 3. These animals had not received the oral drench with the DEEDI inoculum at Sample 2. Other molecular techniques for detecting and counting *S. jonesii* in RF are being investigated.

It is interesting to note that the DNA of the bacteria found in the RF had slightly different combinations of DNA base pairs compared to those of *S. jonesii* produced in the DEEDI inoculum, indicating that the *S. jonesii* detected was a genetically different strain to the *S. jonesii* in the original *in vitro* DEEDI inoculum used. Possible explanations for this genetic change to *S. jonesii* are: 1) the original DEEDI bacteria mutated in the rumen; or 2) the DEEDI bacteria did not persist in the rumen and the different strain of bacteria found came from another source (e.g. the environment from earlier attempts to inoculate cattle or from other carrier cattle).

Liveweight gain

Initial liveweight gain could not be measured prior to Sample 1 because cattle were not weighed (with gutfill) prior to starting to graze the leucaena pasture. Cattle averaged 0.61 kg/hd/d (range 0.29 to 1.11 kg/hd/d) liveweight gain over the second and 0.89 kg/hd/d (range 0.44 to 1.20 kg/hd/d) over the third grazing periods. The research team does not think that the LWG at Sample 3 was affected by leucaena toxicity despite the excretion high levels of 2,3-DHP by a few of the cattle at this time. More research work is required to understand the degree of toxicity of the 2,3-DHP compound.

Summary Table 1 Data from cattle grazing leucaena before (Samples 1 & 2) and after (Sample 3) inoculation with *S. jonesii*.

	Sample 1	Sample 2	Sample 3
Leucaena in diet (%)			
Average	36%	74%	51%
Range	17-45	62-83	42-64
Urine 3,4-DHP concentration (ppm)			
Average	27 ppm	10 ppm	9 ppm
Range	0-356	0-26	4-23
Urine 2,3-DHP concentration (ppm)			
Average	43 ppm	30 ppm	122 ppm
Range	0-191	0-141	20-389
Liveweight gain (kg/hd/d)			
Average	*	0.61	0.89
Range		0.29 to 1.11	0.44 to 1.20

Note - ppm is equal to mg/l

* Cattle were not initially weighed onto the leucaena pasture; therefore weight gain prior to Sample 1 could not be measured.

Property 3

Location: Millmerran

Inoculation history: New leucaena grower - never inoculated cattle

Cattle: 14 weaner Angus×Wagyu steers/heifers (240 kg LW) grazed 70 acres of leucaena.

Sample dates: Cattle started grazing leucaena - 9/2/09

Sample 1 - 2/4/09 (after 53 days grazing leucaena to determine toxicity status)

Sample 2 - 14/5/09 (after 95 days grazing leucaena before inoculation)

Sample 3 - 25/6/09 (after 42 days grazing leucaena following inoculation)

Trial 1 - The impact of leucaena toxicity on animal health & production

The impact of leucaena toxicity on animal health and production was studied in cattle grazing leucaena before and after inoculation with DEEDI *Synergistes jonesii* inoculum following the current industry recommended protocol.

KEY FINDINGS

1. The cattle were **not** protected from leucaena toxicity at the end of Trial 1.
2. The cattle consumed significant amounts (30-69%) of leucaena in their diets throughout the trial, enough to induce toxicity in unprotected animals.
3. Cattle excreted high levels of 3,4-DHP in their urine indicating they were suffering leucaena toxicity throughout the trial, even 42 days after inoculation of the herd with DEEDI inoculum. At each same period 7-9 animals were excreting toxin >300 ppm. The highest concentration recorded was >2000 ppm.
4. DNA tools developed to determine the presence/absence of *S. jonesii* and to count their numbers in rumen fluid (RF) were not sensitive enough to detect the bacteria over 90% of the samples collected in the research program because *S. jonesii* appears to be present in very low numbers. Therefore, changes in bug populations in the rumen of individual animals or the rate of spread between animals could not be monitored. *Synergistes jonesii* was not detected in any RF samples collected from cattle.
5. Bacteria with the same genetic code as the DEEDI inoculum were not detected in RF at Sample 3.
6. This suggests that UQ failed to successfully inoculate the 2 animals drenched with 100 ml of *S. jonesii* DEEDI inoculum.
7. There were no obvious trends in cattle liveweight gain related to toxicity.

Trial 2 - The retention of *S. jonesii* in cattle removed from leucaena

The cattle from Trial 1 were removed from the leucaena pasture immediately following Sample 3 and were placed on an oats pasture for 49 days. They were sold prior to being reintroduced to leucaena to determine if *S. jonesii* had survived.

KEY FINDINGS

1. The rate of decline in *S. jonesii* populations could not be monitored in the herd because of lack of enough sensitivity of current DNA tools of measurement.
2. *S. jonesii* DNA was detected in only 1 of the 48 RF samples collected during the trial.

MANAGEMENT RECOMMENDATIONS

1. Inoculate cattle grazing leucaena with DEEDI *S. jonesii* bacteria. Follow the current recommended protocol by inoculating 10% of the herd, 2 weeks after they start consuming leucaena.
2. Maintain 'carrier' animals on leucaena pastures throughout winter to ensure *S. jonesii* is maintained in their rumen and can then spread throughout a new herd when introduced to leucaena the following spring/summer.
3. Routinely check the toxicity status of 'carrier' and production animals each year using the urine test kit to ensure your herd is protected.

MORE DETAILED ANALYSIS

Trial 1 - The impact of leucaena toxicity on animal health & production

Indicators of toxicity were measured in 15 head before and after inoculation with *S. jonesii*.

Amount of leucaena in diet

The proportion of leucaena in the diet selected by the cattle was estimated by analysing faeces samples using the delta carbon method. The average amount of leucaena in the diet of the cattle varied from 30% at the start of the experiment to 69% at the end of the study period. At these levels of leucaena intake we would expect unprotected animals to excrete significant quantities of the DHP toxins. It is also important to note that cattle only need 35-40% leucaena in diet to achieve 1 kg/hd/d liveweight gain. The lower level of leucaena intake at Sample 1 may have been due to the fact that the cattle periodically escaped from the leucaena paddock into adjoining grass or crop stubble areas. If the cattle had escaped in the 3-7 days prior to Sample 1, then the 'grass' content of the diet would be higher than expected from grass:leucaena ratio on offer in the paddock.

Toxicity status of the herd

Urine

The concentration of the toxins 3,4-DHP and 2,3-DHP was measured in the urine samples by high performance liquid chromatography. Unprotected cattle grazing leucaena excrete these toxins in concentrations exceeding 200 ppm (or mg/l). Cattle excreted high levels of 3,4-DHP at Sample 1 (average = 491 ppm), Sample 2 (average = 521) and Sample 3 (average = 310). At each sample period, 12, 9 and 9 animals had 3,4-DHP concentrations in their urine exceeding 200 ppm. On 3 occasions individual animals had urinary 3,4-DHP concentrations exceeding 1000 ppm. The concentration of 2,3-DHP in urine was only found to be high at Sample 1 when 5 out of the 14 trial animals had concentrations exceeding 200 ppm. Throughout the rest of the trial cattle excreted very little 2,3-DHP indicating that effective strains of *S. jonesii* were not present in the rumen of these animals.

Blood

Very low concentrations of the toxins (<20 ppm) were detected in the blood of the animals throughout the experiment. There was no relationship between blood and urine DHP concentrations. These findings indicate that measuring blood toxin concentrations will be of little use in determining the toxicity status of animals.

Rumen fluid

Two DNA tests were conducted on the rumen fluid (RF) samples. The first method tried to detect whether *S. jonesii* DNA was present in the RF. The second method tried to count the numbers of *S. jonesii* bacteria present in the RF. The trial found that *S. jonesii* was present in very low numbers ($<10^5$ bacteria/ml) in the RF of cattle grazing leucaena pastures compared to other common rumen bacteria.

These new techniques developed to detect and count *S. jonesii* were not sensitive enough to detect any bacteria in the cattle tested. CSIRO then developed an even more sensitive DNA detection tool and it detected *S. jonesii* in none of the RF samples collected from the cattle. Other molecular techniques for detecting and counting *S. jonesii* in RF are being investigated.

Liveweight gain

Over the 137 days of the trial cattle gained an average of 0.70 kg/hd/d. Cattle averaged 0.93 kg/hd/d (range 0.66 to 1.21 kg/hd/d) liveweight gain over the first grazing period, 0.95 kg/hd/d (range 0.57 to 1.17 kg/hd/d) over the second and 0.15 kg/hd/d (range -0.31 to 0.50 kg/hd/d) over the third. The poor performance over the last grazing period could have been due to the cumulative effect of DHP toxicity; however it also coincided with a period of 2 weeks of cold wet miserable weather which adversely affected the LWG of other trial cattle in the Millmerran district.

Summary Table 1 Data from cattle grazing leucaena before (Samples 1 & 2) and after (Sample 3) inoculation with *S. jonesii*.

	Sample 1	Sample 2	Sample 3
Leucaena in diet (%)			
Average	30%	69%	69%
Range	20-55	64-72	50-76
Urine 3,4-DHP concentration (ppm)			
Average	491 ppm	521 ppm	310 ppm
Range	163-1055	88-2018	8-734
Urine 2,3-DHP concentration (ppm)			
Average	224 ppm	19 ppm	44 ppm
Range	10-750	0-77	12-180
Liveweight gain (kg/hd/d)			
Average	0.93	0.95	0.15
Range	0.66-1.21	0.57-1.17	-0.31 to 0.50

Note - ppm is equal to mg/l

Trial 2 - The retention of *S. jonesii* in cattle removed from leucaena

Eight (8) cattle from Trial 1 were removed from the leucaena pasture immediately following Sample 3 (25/6/09) and were placed on to an oats cv. Taipan pasture. The population of *S. jonesii* was monitored in rumen fluid (RF) samples from cattle grazing oats for 7, 14, 21, 28, 35 and 49 days. The Wagyu × cattle were then sent to a feedlot for long-term grain feeding and therefore could not be reintroduced to leucaena to determine if *S. jonesii* had survived.

Population dynamics of *S. jonesii* in the rumen

Due to the problem of not having enough sensitivity in our DNA analysis, it was not possible to monitor changes in bug populations over time grazing oats as we had hoped. *Synergistes jonesii* DNA was detected in only 1 of the 48 RF samples taken from the animals over the 6 sample dates (tag #12 at 21 days off leucaena).

It is interesting to note that the DNA of the bacteria found in the RF of this animal had slightly different combinations of DNA base pairs compared to those of *S. jonesii* produced in the DEEDI inoculum, indicating that the *S. jonesii* detected was a genetically different strain to the *S. jonesii* in the original *in vitro* DEEDI inoculum used. A possible explanation for the genetic difference in the *S. jonesii* strains found in the RF samples is that it is a naturalized variant of the DEEDI inoculum that was present in the environment and colonized the rumen of animal tag #12.

Property 4

Location: Millmerran

Inoculation history: New leucaena grower - never inoculated cattle

Cattle: 29 Santa/Brahman × heifers (335 kg LW) grazed 80 acres of leucaena

Sample dates: Cattle started grazing leucaena - 3/3/09

Sample 1 - 2/4/09 (after 30 days grazing leucaena to determine toxicity status)

Sample 2 - 14/5/09 (after 72 days grazing leucaena before inoculation)

Sample 3 - 25/6/09 (after 42 days grazing leucaena following inoculation)

The impact of leucaena toxicity on animal health & production

The impact of leucaena toxicity on animal health and production was studied in cattle grazing leucaena before and after inoculation with DEEDI *Synergistes jonesii* inoculum following the current industry recommended protocol.

KEY FINDINGS

1. The cattle were suffering leucaena toxicity at the beginning of the trial.
2. The cattle were protected from leucaena toxicity at the end of the trial.
3. The cattle consumed high levels (55-60%) of leucaena in their diets throughout the trial; high enough to induce toxicity in unprotected animals.
4. Cattle excreted high levels of both 3,4-DHP & 2,3-DHP in their urine indicating they were suffering leucaena toxicity after the 30 day period ending at Sample 1 and high levels of 3,4-DHP only at Sample 2. Following inoculation of the herd with DEEDI inoculum low levels of both 3,4-DHP and 2,3-DHP were observed in the urine samples.
5. *Synergistes jonesii* bacteria were only detected in 2 of the RF samples collected throughout the trial.
6. Bacteria with the same genetic code as the DEEDI inoculum were not detected in RF at Sample 3.
7. We assume very low numbers of *S. jonesi*i were present in the rumen fluid (RF) samples given DHP was being detoxified by the cattle. DNA tools developed to determine the presence/absence of *S. jonesii* and to count their numbers in RF were not sensitive enough to enable changes in bug populations in the rumen of individual animals or the rate of spread between animals to be monitored.
8. There were no obvious trends in cattle liveweight gain related to toxicity.

MANAGEMENT RECOMMENDATIONS

1. Inoculate cattle grazing leucaena with DEEDI *S. jonesii* bacteria. Follow the current recommended protocol by inoculating 10% of the herd, 2 weeks after they start consuming leucaena.
2. Maintain 'carrier' animals on leucaena pastures throughout winter to ensure *S. jonesii* is maintained in their rumen and can then spread throughout a new herd when introduced to leucaena the following spring/summer.
3. Routinely check the toxicity status of 'carrier' and production animals annually using the urine test kit to ensure your herd is protected.

MORE DETAILED ANALYSIS

The impact of leucaena toxicity on animal health & production

Indicators of toxicity were measured in 15 head before and after inoculation with *S. jonesii*.

Amount of leucaena in diet

The proportion of leucaena in the diet selected by the cattle was estimated by analysing faeces samples using the delta carbon method. The average amount of leucaena in the diet of the cattle was steady at 55-60% throughout the experiment. At these high levels of leucaena intake we would expect unprotected animals to excrete significant quantities of the DHP toxins. It is also important to note that cattle need 35-40% leucaena in diet to achieve 1 kg/hd/d liveweight gain.

Toxicity status of the herd

Urine

The concentration of the toxins 3,4-DHP and 2,3-DHP was measured in the urine samples by high performance liquid chromatography. Unprotected cattle grazing leucaena excrete these toxins in concentrations exceeding 200 ppm (or mg/l). *C*attle excreted high levels of 3,4-DHP (average = 664 ppm) and 2,3-DHP (average = 294 ppm) at Sample 1, with 10 of 12 and 6 of 12 animals exceeding the toxicity threshold of 200 ppm for 3,4-DHP and 2,3-DHP respectively. At Sample 2 the cattle were still excreting high levels of 3,4-DHP (average = 686 ppm) 72 days after they started grazing leucaena, while very little 2,3-DHP was being excreted. After inoculation of the cattle with DEEDI inoculum, animals excreted low concentrations of both toxins as was expected.

Blood

Very low concentrations of the toxins 3,4-DHP and 2,3-DHP (<10 ppm) were detected in the blood of the animals throughout the experiment. There was no relationship between blood and urine DHP concentrations. These findings indicate that measuring blood toxin concentrations will be of little use in determining the toxicity status of animals.

Rumen fluid

Two DNA tests were conducted on the rumen fluid (RF) samples. The first method tried to detect whether *S. jonesii* DNA was present in the RF. The second method tried to count the numbers of *S. jonesii* bacteria present in the RF. The trial found that *S. jonesii* was present in very low numbers ($<10^5$ bacteria/ml) in the RF of cattle grazing leucaena pastures compared to other common rumen bacteria. Even at these low populations, *S. jonesii* appeared to prevent leucaena toxicity.

The new DNA techniques developed to detect and count *S. jonesii* were not sensitive enough to detect any bacteria in the cattle tested. CSIRO then developed an even more sensitive DNA detection tool. It could detect *S. jonesii* in 2 RF samples: animal tag# 285 and animal tag# 875 at Sample 3. Animal # 285 had received the oral drench with the DEEDI inoculum at Sample 2, while animal # 875 had not. Other molecular techniques for detecting and counting *S. jonesii* in RF are being investigated.

It is interesting to note that the DNA of the bacteria found in the RF of both cattle had slightly different combinations of DNA base pairs compared to those of *S. jonesii* produced in the DEEDI inoculum, indicating that the *S. jonesii* detected was a genetically different strain to the *S. jonesii* in the original *in vitro* DEEDI inoculum used. Possible explanations for this genetic change to *S. jonesii* are: 1) the original DEEDI bacteria mutated in the rumen; or 2) the DEEDI bacteria did not persist in the rumen and the different strain of bacteria found came from another source (e.g. the environment or other carrier cattle).

Liveweight gain

Initial liveweight gain could not be determined over the first grazing period because the cattle were purchased and arrived empty so gutfill could not be accounted for at the first trial weighing at Sample 1. Cattle averaged, 0.31 kg/hd/d (range -0.69 to 0.88 kg/hd/d) liveweight gain over the second grazing period and 0.22 kg/hd/d (range -1.42 to 0.74 kg/hd/d) over the third. The research team does not think that the LWG at Sample 3 was affected by leucaena toxicity due to the absence of toxin excretion by the cattle at this time. The poor performance over the last grazing period coincided with a shortage of feed supply (both leucaena and roughage) and a period of 2 weeks of cold wet miserable weather which adversely affected the LWG of other trial cattle in the Millmerran district.

Summary Table 1 Data from cattle grazing leucaena before (Samples 1 & 2) and after (Sample 3) inoculation with *S. jonesii*.

	Sample 1	Sample 2	Sample 3
Leucaena in diet (%)			
Average	55%	55%	58%
Range	47-63	48-61	49-68
Urine 3,4-DHP concentration (ppm)			
Average	664 ppm	686 ppm	56 ppm
Range	107-1579	177-1182	9-119
Urine 2,3-DHP concentration (ppm)			
Average	294 ppm	41 ppm	2 ppm
Range	14-831	8-135	0-14
Liveweight gain (kg/hd/d)			
Average	*	0.31	0.22
Range		-0.69 to 0.88	-1.42 to 0.74

Note - ppm is equal to mg/l

* No initial weight gain could be calculated because the cattle arrived under curfew and were not weighed with gutfill prior to going onto the leucaena.

Property 5

Location: Goondiwindi

Inoculation history: Problem herd - Inoculated cattle in the past but had poor weight gains

Cattle: 150 Brahman steers (285 kg LW) grazed 90 acres of leucaena + 140 acres grass

Sample dates: Cattle started grazing leucaena - 6/1/09

Sample 1 - 23/1/09 (after 17 days grazing leucaena to determine toxicity status)

Sample 2 - 20/3/09 (after 73days grazing leucaena before inoculation)

Sample 3 - 7/5/09 (after 48 days grazing leucaena following inoculation)

Trial 1 - The impact of leucaena toxicity on animal health & production

The impact of leucaena toxicity on animal health and production was studied in cattle grazing leucaena before and after inoculation with DEEDI *Synergistes jonesii* inoculum following the current industry recommended protocol.

KEY FINDINGS

1. The cattle were protected from leucaena toxicity at the end of Trial 1.
2. The cattle consumed moderate amounts (18-41%) of leucaena in their diets throughout the trial. The diets consumed at Sample 2 & 3 (average 18 & 26% respectively) are probably high enough to induce toxicity in unprotected animals.
3. Cattle excreted high levels of 3,4-DHP in their urine indicating they were suffering leucaena toxicity during the 17 day period ending at Sample 1. Surprisingly, by Sample 2 the cattle appeared to have gained protection (that is they acquired DHP degrading rumen micro-organisms) before UQ inoculated the herd with DEEDI inoculum, as evidenced by low levels of both 3,4-DHP and 2,3-DHP in the urine samples.
4. It is possible that as these cattle were purchased they may have had previous exposure to cattle inoculated with *S. jonesii*. Alternatively, resident bacteria present in the environment (persisting from earlier attempts to inoculate cattle) may have successfully colonized rumens of the trial cattle.
5. Very low numbers of *S. jonesi*i were found in the rumen fluid (RF) samples. DNA tools developed to determine the presence/absence of *S. jonesii* and to count their numbers in RF were not sensitive enough to enable changes in bug populations in the rumen of individual animals or the rate of spread between animals to be monitored.
6. *Synergistes jonesii* bacteria were detected in the RF of only 11 animals (animal #14, 45, 69, 80, 92, 101, 102, 105, 167, 202 & 206) at Samples 1 & 2 **before** inoculation with DEEDI inoculum. All these positive DNA test results were weak indicating they were

present in low numbers. The *S. jonesii* bacteria in all samples were genetically different to the strains present in the DEEDI inoculum used.

7. Bacteria with the same genetic code as the DEEDI inoculum were not detected in RF at Sample 3.

8. There were no obvious trends in cattle liveweight gain related to toxicity.

Trial 2 - The retention of *S. jonesii* in cattle removed from leucaena

The cattle from Trial 1 were removed from the leucaena pasture immediately following Sample 3 and were placed on a native grass pasture with water medication for 120 days. The ability of *S. jonesii* to persist in the rumen was studied by placing the cattle back on leucaena for 21 days before checking them for signs of toxicity.

KEY FINDINGS

1. Cattle retained viable populations of *S. jonesii* after grazing native grass pasture with water medication for 120 days in Trial 2.

2. The rate of decline in *S. jonesii* populations could not be monitored because they exist in low populations in the rumen, below the level of sensitivity of current DNA tools of measurement.

MANAGEMENT RECOMMENDATIONS

1. Inoculate cattle grazing leucaena with DEEDI *S. jonesii* bacteria. Follow the current recommended protocol by inoculating 10% of the herd, 2 weeks after they start consuming leucaena.

2. Maintain 'carrier' animals on leucaena pastures throughout winter to ensure *S. jonesii* is maintained in their rumen and can then spread throughout a new herd when introduced to leucaena the following spring/summer.

3. Routinely check the toxicity status of 'carrier' and production animals annually using the urine test kit to ensure your herd is protected.

MORE DETAILED ANALYSIS

Trial 1 - The impact of leucaena toxicity on animal health & production

Indicators of toxicity were measured in 15 head before and after inoculation with *S. jonesii*.

Amount of leucaena in diet

The proportion of leucaena in the diet selected by the cattle was estimated by analysing faeces samples using the delta carbon method. The average amount of leucaena in the diet of the cattle varied from 41% at the start of the experiment to 18 & 26% at Sample dates 2 & 3 respectively. At these levels of leucaena intake we would expect unprotected animals to

excrete significant quantities of the DHP toxins. It is also important to note that cattle need 35-40% leucaena in diet to achieve 1 kg/hd/d liveweight gain.

Toxicity status of the herd

Urine

The concentration of the toxins 3,4-DHP and 2,3-DHP was measured in the urine samples by high performance liquid chromatography. Unprotected cattle grazing leucaena excrete these toxins in concentrations exceeding 200 ppm (or mg/l). Cattle excreted high levels of 3,4-DHP at Sample 1 (average = 376 ppm), with 9 of the 15 animals exceeding the toxicity threshold of 200 ppm. At this time cattle also excreted moderate levels of 2,3-DHP (average = 272 ppm), with 7 animals exceeding the toxicity threshold of 200 ppm. This indicated that *S. jonesii* may have been present in the rumen of these animals but was not efficiently degrading the toxins. All cattle were excreting very little 3,4-DHP at Sample 2 (73 days after they started grazing leucaena) prior to inoculation with the DEEDI inoculum. It would appear that the cattle somehow picked up rumen microbes capable of degrading the toxin. After inoculation of the cattle with DEEDI inoculum, animals continued to excrete undetectable concentrations of both toxins as was expected.

Blood

Very low concentrations of the toxins 3,4-DHP and 2,3-DHP (<5 ppm) were detected in the blood of the animals throughout the experiment. There was no relationship between blood and urine DHP concentrations. These findings indicate that measuring blood toxin concentrations will be of little use in determining the toxicity status of animals.

Rumen fluid

Two DNA tests were conducted on the rumen fluid (RF) samples. The first method tried to detect whether *S. jonesii* DNA was present in the RF. The second method tried to count the numbers of *S. jonesii* bacteria present in the RF. The trial found that *S. jonesii* was present in very low numbers ($<10^5$ bacteria/ml) in the RF of cattle grazing leucaena pastures compared to other common rumen bacteria. Even at these low populations, *S. jonesii* appeared to prevent leucaena toxicity.

The new DNA techniques developed to detect and count *S. jonesii* were not sensitive enough to detect any bacteria in the cattle tested. CSIRO then developed an even more sensitive DNA detection tool and it detected *S. jonesii* in only 11 animals at one sample date as follows: animals tag #14, 45, 80, 101, 102, 105, 167, 202 & 206 at Sample 1; and animals tag# 69 & 92 at Sample 2. None of these animals had received the oral drench with the DEEDI inoculum. Other molecular techniques for detecting and counting *S. jonesii* in RF are being investigated.

It is interesting to note that the DNA of the bacteria found in the RF of all the animals had slightly different combinations of DNA base pairs compared to those of *S. jonesii* produced in the DEEDI inoculum, indicating that the *S. jonesii* detected was a genetically different strain to the *S. jonesii* in the original *in vitro* inoculum used. A possible explanation for the genetic difference in the *S. jonesii* strains found in the RF samples is that it is a naturalized variant of

the DEEDI inoculum that has carried over in the environment from earlier inoculation attempts.

Liveweight gain

Over the 121 days of the trial cattle gained an average of 0.75 kg/hd/d. Cattle averaged 0.44 kg/hd/d (range 0 to 0.88 kg/hd/d) liveweight gain over the first grazing period, 0.76 kg/hd/d (range 0.54 to 0.92 kg/hd/d) over the second and 0.87 kg/hd/d (range 0.50-1.17 kg/hd/d) over the third. Note that cattle were weighed after curfew at Sample 1, so the LWG observed at Sample 2 would be an overestimate including gutfill and water. The research team does not think that LWG was affected by leucaena toxicity during the trial due to the absence of toxin excretion in cattle at Samples 2 & 3.

Summary Table 1 Data from cattle grazing leucaena before (Samples 1 & 2) and after (Sample 3) inoculation with *S. jonesii*.

	Sample 1	Sample 2	Sample 3
Leucaena in diet (%)			
Average	41%	18%	26%
Range	28-49	11-28	20-34
Urine 3,4-DHP concentration (ppm)			
Average	376 ppm	78 ppm	0 ppm
Range	91-964	1-248	-
Urine 2,3-DHP concentration (ppm)			
Average	272 ppm	39 ppm	0 ppm
Range	44-647	0-95	-
Liveweight gain (kg/hd/d)			
Average	0.44	0.76*	0.87
Range	0-0.88	0.54-0.92	0.50-1.17

Note - ppm is equal to mg/l

* Liveweight gain may be overestimated at Sample 2 due to cattle being weighed after curfew at Sample 1 only and not thereafter.

Trial 2 - The retention of *S. jonesii* in cattle removed from leucaena

Eight (8) cattle from the toxicity study were removed from the leucaena pasture on the 17/06/09 and were placed on to a native grass pasture with water medication. The population of *S. jonesii* was monitored in rumen fluid (RF) samples after 0, 7, 15, 29, 43, 57, 92 and 120 days as the cattle grazed the grass pasture. After 120 days (15/10/09) the cattle were returned to a leucaena pasture for 21 days before blood, urine, faeces and RF samples were taken on 5/11/09 to determine if *S. jonesii* had survived in the absence of leucaena in the cattle's diet.

Population dynamics of *S. jonesii* in the rumen

Due to the problem of not having enough sensitivity in our DNA analysis, it was not possible to monitor changes in bug populations over time grazing native grass pasture as we had hoped. *Synergistes jonesii* DNA was not detected in any of the 64 RF samples taken from the 8 animals over the 8 sample dates.

Amount of leucaena in diet

Once the cattle had been returned to a leucaena pasture, the proportion of leucaena in diet averaged 29% (range 23-35%), see Summary Table 2 below. These levels of leucaena intake would have enabled *S. jonesii* populations to build up in the rumen of cattle had they survived the period of absence of leucaena in diet.

Toxicity status of the herd

Urine

Urine analysis indicated that the cattle were protected from leucaena toxicity 3 weeks after they resumed eating leucaena. Animals were excreting negligible quantities of 3,4-DHP & 2,3-DHP.

Blood

Blood toxin concentrations were not detectable as observed in Trial 1, and were not a useful indicator of the toxicity status of the animals.

Summary Table 2 Data from cattle fed leucaena after 120 days grazing native grass pasture with water medication

	After 21 days back on leucaena
Leucaena in diet (%)	
Average	29%
Range	23-35
Urine 3,4-DHP concentration (ppm)	
Average	2 ppm
Range	0-14
Urine 2,3-DHP concentration (ppm)	
Average	0 ppm
Range	-

Note - ppm is equal to mg/l

Property 6

Location: Kaimkillenbun

Inoculation history: New herd - cattle had no previous inoculation history

Cattle: 25 maiden Angus heifers preg. tested in calf (460 kg LW) rotationally grazed >200 acres of leucaena and grass pasture

Sample dates: Cattle started grazing leucaena - 2/12/08

Sample 1 - 11/2/09 (after 71 days grazing leucaena to determine toxicity status)

Sample 2 - 31/3/09 (after 119 days grazing leucaena before inoculation)

Sample 3 - 26/5/09 (after 56 days grazing leucaena following inoculation)

Trial 1 - The impact of leucaena toxicity on animal health & production

The impact of leucaena toxicity on animal health and production was studied in cattle grazing leucaena before and after inoculation with DEEDI *Synergistes jonesii* inoculum following the current industry recommended protocol.

KEY FINDINGS

1. Cattle were suffering from 2,3-DHP toxicity at the beginning of Trial 1.
2. The cattle were protected from leucaena toxicity at the end of Trial 1.
3. The cattle consumed high amounts (60-70%) of leucaena in their diets throughout the trial, enough to induce toxicity in unprotected animals.
4. Throughout the trial cattle did not excrete toxic concentrations of 3,4-DHP. However, cattle did excrete very high levels of 2,3-DHP in their urine indicating they were suffering leucaena toxicity during the 105 day period ending at Sample 1. Surprisingly, by Sample 2 the cattle appeared to be gaining protection (that is they acquired DHP degrading rumen micro-organisms) before UQ inoculated the herd with DEEDI inoculum, as evidenced by lower levels of 2,3-DHP in the urine samples.
5. It is possible that as these cattle may have had previous exposure to cattle inoculated with *S. jonesii* (e.g. from purchased bulls and cows entering the stud) even though no deliberate attempt to inoculate the cattle had been made. This strain of bacteria appeared to be relatively inefficient at degrading 2,3-DHP.
6. Very low numbers of *S. jonesi*i were found in the rumen fluid (RF) samples. DNA tools developed to determine the presence/absence of *S. jonesii* and to count their numbers in RF were not sensitive enough to enable changes in bug populations in the rumen of individual animals or the rate of spread between animals to be monitored.

7. *Synergistes jonesii* bacteria were detected in only 3 RF samples from 2 animals (animal #C28 at Samples 2 & 3; #C17 at Sample 3) All these positive DNA test results were relatively weak indicating they were present in low numbers. The *S. jonesii* bacteria in all samples were genetically different to the strains present in the DEEDI inoculum used.

8. Bacteria with the same genetic code as the DEEDI inoculum were not detected in RF at Sample 3.

9. There were no obvious trends in cattle liveweight gain related to toxicity.

Trial 2 - The retention of *S. jonesii* in cattle removed from leucaena

The cattle from Trial 1 were removed from the leucaena pasture on the 15/6/09and were placed on either: 1) a tropical grass pasture (*Chloris gayana*/*Dicanthium* spp.); or2) an oats cv. Reil and tropical grass pasture for 112 days. The ability of *S. jonesii* to persist in the rumen was studied by placing the cattle back on leucaena for 21 days before checking them for signs of toxicity.

KEY FINDINGS

1. Cattle retained viable populations of *S. jonesii* after grazing both grass pasture and oats/grass pasture for 112 days in Trial 2.

2. The rate of decline in *S. jonesii* populations could not be monitored because they exist in low populations in the rumen, below the level of sensitivity of current DNA tools of measurement.

3. *Synergistes jonesii* could only be detected in3 RF samples from 2 animals in the oat-fed cattle. DNA sequences of these bacteria differed from the DEEDI *S. jonesii* inoculum.

MANAGEMENT RECOMMENDATIONS

1. Inoculate cattle grazing leucaena with DEEDI *S. jonesii* bacteria. Follow the current recommended protocol by inoculating 10% of the herd, 2 weeks after they start consuming leucaena.

2. Maintain 'carrier' animals on leucaena pastures throughout winter to ensure *S. jonesii* is maintained in their rumen and can then spread throughout a new herd when introduced to leucaena the following spring/summer.

3. Routinely check the toxicity status of 'carrier' and production animals annually using the urine test kit to ensure your herd is protected.

MORE DETAILED ANALYSIS

Trial 1 - The impact of leucaena toxicity on animal health & production

Indicators of toxicity were measured in 15 head before and after inoculation with *S. jonesii*.

Amount of leucaena in diet

The proportion of leucaena in the diet selected by the cattle was estimated by analysing faeces samples using the delta carbon method. The average amount of leucaena in the diet of the cattle varied from 63% at the start of the experiment to 70% at the end of Trial 1. At these levels of leucaena intake we would expect unprotected animals to excrete significant quantities of the DHP toxins. It is also important to note that cattle need 35-40% leucaena in diet to achieve 1 kg/hd/d liveweight gain.

Toxicity status of the herd

Urine

The concentration of the toxins 3,4-DHP and 2,3-DHP was measured in the urine samples by high performance liquid chromatography. Unprotected cattle grazing leucaena excrete these toxins in concentrations exceeding 200 ppm (or mg/l). Throughout the trial the cattle excreted low concentrations (<50 ppm) of the toxin 3,4-DHP. However, cattle excreted very high levels of 2,3-DHP at Sample 1 (average = 1036 ppm), with 12 of the 15 animals exceeding the toxicity threshold of 200 ppm. This is unusual and indicated that a strain of *S. jonesii* was present in the rumen of these animals but was not efficiently degrading the 2,3-DHP toxin. The cattle were excreting less 2,3-DHP (average = 145 ppm) at Sample 2 (109 days after they started grazing leucaena) prior to inoculation with the DEEDI inoculum, with 4 of the 15 animals exceeding the toxicity threshold of 200 ppm. At Sample 3, only 1 animal had a urinary 2,3-DHP concentration exceeding the toxicity threshold of 200 ppm. It would appear that the cattle somehow picked up rumen microbes capable of completely degrading both of the DHP toxins. After inoculation of the cattle with DEEDI inoculum, animals continued to excrete undetectable concentrations of both toxins as was expected.

Blood

Very low concentrations of the toxins 3,4-DHP (<10 ppm) and 2,3-DHP (<20 ppm) were detected in the blood of the animals throughout the experiment. There was no relationship between blood and urine DHP concentrations. These findings indicate that measuring blood toxin concentrations will be of little use in determining the toxicity status of animals.

Rumen fluid

Two DNA tests were conducted on the rumen fluid (RF) samples. The first method tried to detect whether *S. jonesii* DNA was present in the RF. The second method tried to count the numbers of *S. jonesii* bacteria present in the RF. The trial found that *S. jonesii* was present in very low numbers ($<10^5$ bacteria/ml) in the RF of cattle grazing leucaena pastures compared to other common rumen bacteria. Even at these low populations, *S. jonesii* appeared to prevent leucaena toxicity.

The new DNA techniques developed to detect and count *S. jonesii* were not sensitive enough to detect any bacteria in the cattle tested. CSIRO then developed an even more sensitive DNA detection tool and it detected *S. jonesii* in only 3 RF samples from 2 different

animals (#C28 at Samples 2 & 3; #C17 at Sample 3). None of these animals had received the oral drench with the DEEDI inoculum. Other molecular techniques for detecting and counting *S. jonesii* in RF are being investigated.

It is interesting to note that the DNA of the bacteria found in the RF of all the animals had slightly different combinations of DNA base pairs compared to those of *S. jonesii* produced in the DEEDI inoculum, indicating that the *S. jonesii* detected in the heifers was a genetically different strain to the *S. jonesii* in the original *in vitro* inoculum used. A possible explanation for this genetic change to *S. jonesii* is that the DEEDI bacteria did not persist in the rumen and the different strain of bacteria found came from another source (e.g. the environment or other carrier cattle).

Liveweight gain

Liveweight gain was not possible to estimate over period 1 because the cattle were not weighed before entering the leucaena pastures. Cattle averaged 0.84 kg/hd/d (range 0.44 to 1.27 kg/hd/d) over the second period and 0.22 kg/hd/d (range -0.14 to 0.50 kg/hd/d) over the third. The research team does not think that LWG was affected by leucaena toxicity during the trial due to the absence of toxin excretion in cattle at Samples 2 & 3. The poor performance over the last grazing period coincided with a period of 2 weeks of cold wet miserable weather. Cattle typically lose weight under these adverse environmental conditions. In the 19 days following the wet weather, cattle gained 1.34 kg/hd/d while grazing the same pasture.

Summary Table 1 Data from heifers grazing leucaena before (Samples 1 & 2) and after (Sample 3) inoculation with *S. jonesii*.

	Sample 1	Sample 2	Sample 3
Leucaena in diet (%)			
Average	63%	70%	70%
Range	40-73	60-79	60-77
Urine 3,4-DHP concentration (ppm)			
Average	8 ppm	16 ppm	13 ppm
Range	0-47	1-41	4-29
Urine 2,3-DHP concentration (ppm)			
Average	1036 ppm	146 ppm	55 ppm
Range	0-4408	0-509	4-458
Liveweight gain (kg/hd/d)			
Average	-	0.84	0.22
Range	-	0.44-1.27	-0.11 to 0.50

Note - ppm is equal to mg/l

Trial 2 - The retention of *S. jonesii* in cattle removed from leucaena

Two mobs of 8 cattle from the toxicity study were removed from the leucaena pasture on the 15/06/09 and were placed on to either: 1) a hayed-off tropical grass pasture (*Chloris gayana* and *Dichanthium* spp.); or 2) an oats pasture cv. Reil. The population of *S. jonesii* was monitored in rumen fluid (RF) samples after 0, 7, 14, 28, 42, 56, 84 and 112 days as the cattle grazed the grass pasture. After 112 days (5/10/09) the cattle were returned to a leucaena pasture for 21 days before blood, urine, faeces and RF samples were taken on 26/10/09 to determine if *S. jonesii* had survived in the absence of leucaena in the cattle's diet.

Grass-fed cattle

Population dynamics of *S. jonesii* in the rumen

Due to the problem of not having enough sensitivity in our DNA analysis, it was not possible to monitor changes in bug populations over time grazing native grass pasture as we had hoped. *Synergistes jonesii* DNA was not detected in any of the 64 RF samples taken from the 8 grass-fed animals over the 8 sample dates.

Amount of leucaena in diet

Once the cattle had been returned to a leucaena pasture, the proportion of leucaena in diet averaged 42% (range 34-47%), see Summary Table 2 below. These levels of leucaena intake would have enabled *S. jonesii* populations to build up in the rumen of cattle had they survived the period of absence of leucaena in diet. This level of leucaena intake would also induce leucaena toxicity in unprotected cattle.

Toxicity status of the herd

Urine

Urine analysis indicated that the cattle were excreting low quantities of 3,4-DHP and 2,3-DHP when they resumed eating leucaena and were therefore protected from leucaena toxicity.

Blood

Blood toxin concentrations were not detectable as observed in Trial 1, and were not a useful indicator of the toxicity status of the animals.

Summary Table 2 Data from cattle fed leucaena after 112 days grazing hayed off tropical grass pasture

	After 21 days back on leucaena
Leucaena in diet (%)	
Average	42%
Range	34-47
Urine 3,4-DHP concentration (ppm)	
Average	10 ppm
Range	1-16
Urine 2,3-DHP concentration (ppm)	
Average	65 ppm
Range	7-132

Note - ppm is equal to mg/l

Oats-fed cattle

Population dynamics of *S. jonesii* in the rumen

Due to the problem of not having enough sensitivity in our DNA analysis, it was not possible to monitor changes in bug populations over time grazing native grass pasture as we had hoped. *Synergistes jonesii* DNA was detected in only 3 of the 64 RF samples taken from the 8 animals over the 8 sample dates (#C28 at days 0 & 14 off leucaena; #C324 at day 7 off leucaena). Animal C#28 had been inoculated with DEEDI inoculum in Trial 1, however the bacteria detected were genetically different from the DEEDI inoculum.

Amount of leucaena in diet

Once the cattle had been returned to a leucaena pasture, the proportion of leucaena in diet averaged 33% (range 30-35%). These levels of leucaena intake would have enabled *S. jonesii* populations to build up in the rumen of cattle had they survived the period of absence of leucaena in diet. This level of leucaena intake would also induce leucaena toxicity in unprotected cattle.

Toxicity status of the herd

Urine

Urine analysis indicated that the cattle were excreting low quantities of 3,4-DHP and 2,3-DHP when they resumed eating leucaena and were therefore protected from leucaena toxicity.

Blood

Blood toxin concentrations were not detectable as observed in Trial 1, and were not a useful indicator of the toxicity status of the animals.

Summary Table 3 Data from cattle fed leucaena after 112 days grazing oats pasture

	After 21 days back on leucaena
Leucaena in diet (%)	
Average	33%
Range	30-35
Urine 3,4-DHP concentration (ppm)	
Average	0 ppm
Range	-
Urine 2,3-DHP concentration (ppm)	
Average	27 ppm
Range	0-96

Note - ppm is equal to mg/l

Property 7

Location: Wallumbilla

Inoculation history: Problem herd - cattle previously inoculated but may have lost protection

Cattle: 66 Santa X and Charbray steers (490 kg LW) grazed 90 ac leucaena + 140 ac grass

Sample dates: Cattle started grazing leucaena - 6/1/09

Sample 1 - No visit made as cattle weights were regularly recorded

Sample 2 - 24/3/09 (after 77 days grazing leucaena before inoculation)

Sample 3 - 8/5/09 (after 45 days grazing leucaena following inoculation)

The impact of leucaena toxicity on animal health & production

The impact of leucaena toxicity on animal health and production was studied in cattle grazing leucaena before and after inoculation with DEEDI *Synergistes jonesii* inoculum following the current industry recommended protocol.

KEY FINDINGS

1. The cattle were assumed to be suffering leucaena toxicity at the beginning of the trial, based upon previous weight gain data and the colorimetric test of spot urine samples. Whilst the colour of the urine tests was not strong it appeared as though the cattle were not consuming large amounts of leucaena given amount of feed on offer in the paddock. HPLC results indicated that only 1 animal had urinary 3,4-DHP and 1 animal urinary 2,3-DHP concentrations exceeding 200 ppm at Sample 2.

2. The cattle were considered protected from leucaena toxicity at the end of the trial, because all cattle had very low concentrations of the toxins in their urine while leucaena intake remained the same as at Sample 2.

3. The cattle consumed low levels (14%) of leucaena in their diets throughout the trial due to persistent dry conditions preventing leucaena growth. These levels would have not been high enough to induce toxicity (symptoms or poor LWG) in unprotected animals.

4. Cattle excreted low levels of both 3,4-DHP & 2,3-DHP in their urine indicating they were not suffering leucaena toxicity after inoculation with the DEEDI inoculum at Sample 3.

5. *Synergistes jonesii* bacteria were only detected in 1 of the RF samples collected throughout the trial.

6. Bacteria with the same genetic code as the DEEDI inoculum were not detected in RF at Sample 3.

7. We assume very low numbers of *S. jonesi*i were present in the rumen fluid (RF) samples, given DHP was being detoxified by the cattle at Sample 3. DNA tools developed to determine the presence/absence of *S. jonesii* and to count their numbers in RF were not sensitive enough to enable changes in bug populations in the rumen of individual animals or the rate of spread between animals to be monitored.

8. There were no obvious trends in cattle liveweight gain related to toxicity.

MANAGEMENT RECOMMENDATIONS

1. Inoculate cattle grazing leucaena with DEEDI *S. jonesii* bacteria. Follow the current recommended protocol by inoculating 10% of the herd, 2 weeks after they start consuming leucaena.

2. Maintain 'carrier' animals on leucaena pastures throughout winter to ensure *S. jonesii* is maintained in their rumen and can then spread throughout a new herd when introduced to leucaena the following spring/summer.

3. Routinely check the toxicity status of 'carrier' and production animals annually using the urine test kit to ensure your herd is protected.

MORE DETAILED ANALYSIS

The impact of leucaena toxicity on animal health & production

Indicators of toxicity were measured in 15 head before and after inoculation with *S. jonesii*.

Amount of leucaena in diet

The proportion of leucaena in the diet selected by the cattle was estimated by analysing faeces samples using the delta carbon method. The average amount of leucaena in the diet of the cattle was 14% throughout the experiment. At these low levels of leucaena intake we would not expect unprotected animals to excrete significant quantities of the DHP toxins.

Toxicity status of the herd

Urine

The concentration of the toxins 3,4-DHP and 2,3-DHP was measured in the urine samples by high performance liquid chromatography. Unprotected cattle grazing leucaena excrete these toxins in concentrations exceeding 200 ppm (or mg/l). Cattle excreted variable levels of 3,4-DHP (average = 47 ppm) and 2,3-DHP (average = 87 ppm) at Sample 2, with 1 of 15 and 1 of 15 animals exceeding the toxicity threshold of 200 ppm for 3,4-DHP and 2,3-DHP respectively. These concentrations were quite high considering the low levels of leucaena intake consumed by the cattle, suggesting the cattle were not protected from toxicity. After inoculation of the cattle with DEEDI inoculum, animals excreted low concentrations of both toxins at Sample 3 as was expected.

Blood

Very low concentrations of the toxins 3,4-DHP and 2,3-DHP (<2 ppm) were detected in the blood of the animals throughout the experiment. There was no relationship between blood and urine DHP concentrations. These findings indicate that measuring blood toxin concentrations will be of little use in determining the toxicity status of animals.

Rumen fluid

Two DNA tests were conducted on the rumen fluid (RF) samples. The first method tried to detect whether *S. jonesii* DNA was present in the RF. The second method tried to count the numbers of *S. jonesii* bacteria present in the RF. The trial found that *S. jonesii* was present in very low numbers ($<10^5$ bacteria/ml) in the RF of cattle grazing leucaena pastures compared to other common rumen bacteria. Even at these low populations, *S. jonesii* appeared to prevent leucaena toxicity.

The new DNA techniques developed to detect and count *S. jonesii* were not sensitive enough to detect any bacteria in the cattle tested. CSIRO then developed an even more sensitive DNA detection tool. It could detect *S. jonesii* in only 1 of the RF samples collected: animal tag# 162 at Sample 3. This animal had not received the oral drench with the DEEDI inoculum at Sample 2. Other molecular techniques for detecting and counting *S. jonesii* in RF are being investigated.

It is interesting to note that the DNA of the bacteria found in the RF had slightly different combinations of DNA base pairs compared to those of *S. jonesii* produced in the DEEDI inoculum, indicating that the *S. jonesii* detected was a genetically different strain to the *S. jonesii* in the original *in vitro* DEEDI inoculum used. Possible explanations for this genetic change to *S. jonesii* are: 1) the original DEEDI bacteria mutated in the rumen; or 2) the DEEDI bacteria did not persist in the rumen and the different strain of bacteria found came from another source (e.g. the environment or other carrier cattle).

Liveweight gain

Cattle averaged 1.10 kg/hd/d (range 0.76 to 1.39 kg/hd/d) liveweight gain over the second and 0.70 kg/hd/d (range 0.16 to 1.07 kg/hd/d) over the third grazing periods. The excellent LWG of the cattle in the first grazing period reflects the fact that the cattle exhausted the supply of leucaena forage prior to Sample 2, as reflected in the low % leucaena in diet. The research team does not think that the LWG at Sample 3 was affected by leucaena toxicity due to: 1) the low level of leucaena intake; & 2) the absence of toxin excretion by the cattle at this time. The lower LWG performance over the last grazing period coincided with a shortage of feed supply (both leucaena and roughage) due to unseasonably dry weather.

Summary Table 1 Data from cattle grazing leucaena before (Sample 2) and after (Sample 3) inoculation with *S. jonesii*.

	Sample 2	Sample 3
Leucaena in diet (%)		
Average	14%	14%
Range	10-19	10-21
Urine 3,4-DHP concentration (ppm)		
Average	47 ppm	11 ppm
Range	2-490	0-99
Urine 2,3-DHP concentration (ppm)		
Average	87 ppm	1 ppm
Range	0-99	0-5
Liveweight gain (kg/hd/d)		
Average	1.10	0.70
Range	0.76 to 1.39	0.16 to 1.07

Note - ppm is equal to mg/l

Property 8

Location: Murgon

Inoculation history: Problem herd - Inoculated cattle in the past but had poor weight gains

Cattle: 32 Droughtmaster/Braford × steers (340 kg LW) grazed 90 acres of leucaena

Sample dates: Cattle started grazing leucaena - 25/2/09

Sample 1 - 1/4/09 (after 35 days grazing leucaena to determine toxicity status)

Sample 2 - 18/5/09 (after 83 days grazing leucaena before inoculation)

Sample 3 - 17/7/09 (after 48 days grazing leucaena following inoculation)

Trial 1 - The impact of leucaena toxicity on animal health & production

The impact of leucaena toxicity on animal health and production was studied in cattle grazing leucaena before and after inoculation with DEEDI *Synergistes jonesii* inoculum following the current industry recommended protocol.

KEY FINDINGS

1. The cattle were suffering leucaena toxicity at the beginning of Trial 1.
2. The cattle were protected from leucaena toxicity at the end of Trial 1.
3. The cattle consumed high levels (40-70%) of leucaena in their diets throughout the trial; high enough to induce toxicity in unprotected animals.
4. Cattle excreted high levels of 3,4-DHP in their urine indicating they were suffering leucaena toxicity after the 35 day period ending at Sample 1. Surprisingly, by Sample 2 the cattle appeared to have gained protection (that is they acquired DHP degrading rumen micro-organisms) before UQ inoculated the herd with DEEDI inoculum, as evidenced by low levels of both 3,4-DHP and 2,3-DHP in the urine samples.
5. It is possible that as these cattle were purchased they may have had previous exposure to cattle inoculated with *S. jonesii*. Alternatively, resident bacteria present the environment (persisting from earlier attempts to inoculate cattle) may have successfully colonized the rumens of the trial cattle.
6. No *S. jonesii* bacteria were detected in any of the RF samples collected throughout Trial 1.
7. We assume very low numbers of *S. jonesi*i were present in the rumen fluid (RF) samples given DHP was being detoxified by the cattle. DNA tools developed to determine the presence/absence of *S. jonesii* and to count their numbers in RF were not sensitive enough to enable changes in bug populations in the rumen of individual animals or the rate of spread between animals to be monitored.
8. There were no obvious trends in cattle liveweight gain related to toxicity.

Trial 2 - The retention of *S. jonesii* in cattle removed from leucaena

The cattle from Trial 1 were removed from the leucaena pasture immediately following Sample 3 and were placed on an oats/tropical grass pasture for 80 days. The ability of *S. jonesii* to persist in the rumen was studied by placing the cattle back on leucaena for 21 days before checking them for signs of toxicity.

KEY FINDINGS

1. Cattle did not retain viable populations of fully effective strains *S. jonesii* after grazing oats/grass pasture for 80 days in Trial 2.
2. After 3 weeks grazing leucaena cattle were still excreting high levels of 2,3-DHP.
3. The rate of decline in *S. jonesii* populations could not be monitored because they exist in low populations in the rumen, below the level of sensitivity of current DNA tools of measurement.
4. Bacteria with the same genetic code as the DEEDI inoculum used in Trial 1 were not detected in RF collected in Trial 2.

MANAGEMENT RECOMMENDATIONS

1. Inoculate cattle grazing leucaena with DEEDI *S. jonesii* bacteria. Follow the current recommended protocol by inoculating 10% of the herd, 2 weeks after they start consuming leucaena.
2. Maintain 'carrier' animals on leucaena pastures throughout winter to ensure *S. jonesii* is maintained in their rumen and can then spread throughout a new herd when introduced to leucaena the following spring/summer.
3. Routinely check the toxicity status of 'carrier' and production animals annually using the urine test kit to ensure your herd is protected.

MORE DETAILED ANALYSIS

Trial 1 - The impact of leucaena toxicity on animal health & production

Indicators of toxicity were measured in 15 head before and after inoculation with *S. jonesii*.

Amount of leucaena in diet

The proportion of leucaena in the diet selected by the cattle was estimated by analysing faeces samples using the delta carbon method. The average amount of leucaena in the diet of the cattle varied from about 70% at the start of the experiment to 41% at Sample 3. At these high levels of leucaena intake we would expect unprotected animals to excrete significant quantities of the DHP toxins. It is also important to note that cattle need 35-40% leucaena in diet to achieve 1 kg/hd/d liveweight gain.

Toxicity status of the herd

Urine

The concentration of the toxins 3,4-DHP and 2,3-DHP was measured in the urine samples by high performance liquid chromatography. Unprotected cattle grazing leucaena excrete these toxins in concentrations exceeding 200 ppm (or mg/l). Cattle excreted high levels of 3,4-DHP at Sample 1 (average = 217 ppm), with 3 of the 15 animals exceeding the toxicity threshold of 200 ppm. At this time cattle were excreting low levels of 2,3-DHP. All cattle were excreting very little 3,4-DHP at Sample 2 (83 days after they started grazing leucaena) prior to inoculation with the DEEDI inoculum. It would appear that the cattle somehow picked up rumen microbes capable of degrading the toxin. After inoculation of the cattle with DEEDI inoculum, animals continued to excrete undetectable concentrations of both toxins as was expected.

Blood

Very low concentrations of the toxins 3,4-DHP and 2,3-DHP (<5 ppm) were detected in the blood of the animals throughout the experiment. There was no relationship between blood and urine DHP concentrations. These findings indicate that measuring blood toxin concentrations will be of little use in determining the toxicity status of animals.

Rumen fluid

Two DNA tests were conducted on the rumen fluid (RF) samples. The first method tried to detect whether *S. jonesii* DNA was present in the RF. The second method tried to count the numbers of *S. jonesii* bacteria present in the RF. The trial found that *S. jonesii* was present in very low numbers ($<10^5$ bacteria/ml) in the RF of cattle grazing leucaena pastures compared to other common rumen bacteria. Even at these low populations, *S. jonesii* appeared to prevent leucaena toxicity.

The new DNA techniques developed to detect and count *S. jonesii* were not sensitive enough to detect any bacteria in the cattle tested. CSIRO then developed an even more sensitive DNA detection tool; however it too could not detect the presence of *S. jonesii* in the samples. Other molecular techniques for detecting and counting *S. jonesii* in RF are being investigated.

Liveweight gain

Over the 144 days of the trial cattle gained an average of 0.81 kg/hd/d. Cattle averaged 0.75 kg/hd/d (range 0.27 to 1.46 kg/hd/d) liveweight gain over the first grazing period, 0.95 kg/hd/d (range 0.54 to 1.31 kg/hd/d) over the second and 0.73 kg/hd/d (range 0.28 to 0.93 kg/hd/d) over the third. The research team does not think that LWG was affected by leucaena toxicity during the trial due to the absence of toxin excretion by the cattle at Samples 2 & 3.

Summary Table 1 Data from cattle grazing leucaena before (Samples 1 & 2) and after (Sample 3) inoculation with *S. jonesii*.

	Sample 1	Sample 2	Sample 3
Leucaena in diet (%)			
Average	69%	72%	41%
Range	51-80	53-80	31-50
Urine 3,4-DHP concentration (ppm)			
Average	217 ppm	37 ppm	6 ppm
Range	45-635	0-282	0-45
Urine 2,3-DHP concentration (ppm)			
Average	0 ppm	1 ppm	1 ppm
Range	-	0-8	0-7
Liveweight gain (kg/hd/d)			
Average	0.75	0.95	0.73
Range	0.27-1.46	0.54-1.33	0.28-0.93

Note - ppm is equal to mg/l

Trial 2 - The retention of *S. jonesii* in cattle removed from leucaena

Eight (8) cattle from Trial 1 were removed from the leucaena pasture on the 17/07/09 and were placed on to an oats cv. Culgoa II/grass pasture. The population of *S. jonesii* was monitored in rumen fluid (RF) samples after 0, 3, 10, 17, 24, 31, 52 and 80 days as the cattle grazed the oats/grass pasture. After 80 days (5/10/09) the cattle were returned to a leucaena pasture for 21 days before blood, urine, faeces and RF samples were taken on 26/10/09 to determine if *S. jonesii* had survived in the absence of leucaena in the cattle's diet.

Population dynamics of *S. jonesii* in the rumen

Due to the problem of not having enough sensitivity in our DNA analysis, it was not possible to monitor changes in bug populations over time grazing native grass pasture as we had hoped. *Synergistes jonesii* DNA was detected in only 2 of the 64 RF samples taken from the 8 animals over the 8 sample dates. Rumen fluid from animal tag #211 on day 10 off leucaena and animal tag #228 on day 3 off leucaena contained *S. jonesii* DNA.

It is interesting to note that the DNA of the bacteria found in the RF of these animals had slightly different combinations of DNA base pairs compared to those of *S. jonesii* produced in the DEEDI inoculum, indicating that the *S. jonesii* detected was a genetically different strain to the *S. jonesii* in the original *in vitro* DEEDI inoculum used. A possible explanation for the genetic difference in the *S. jonesii* strains found in the RF samples is that it is a naturalized

variant of the DEEDI inoculum that has carried over in the environment from earlier inoculation attempts or has been introduced by carrier cattle (e.g. from the Larsen's heifers).

Amount of leucaena in diet

Once the cattle had been returned to a leucaena pasture, the proportion of leucaena in diet averaged 46% (range 29-56%), see Summary Table 2 below. These levels of leucaena intake would have enabled *S. jonesii* populations to build up in the rumen of cattle had they survived the period of absence of leucaena in diet.

Toxicity status of the herd

Urine

Urine analysis indicated that the cattle were not protected from leucaena toxicity 3 weeks after they resumed eating leucaena. Animals were excreting some 3,4-DHP (average = 118 ppm) with 2 of the 8 animals having concentrations exceeding the toxicity threshold of 200 ppm. Most of the cattle were excreting high levels of 2,3-DHP in their urine (mean = 490 ppm) with 6 of the 8 animals excreting concentrations exceeding the toxicity threshold of 200 ppm.

Blood

Blood toxin concentrations were not detectable as observed in Trial 1, and were not a useful indicator of the toxicity status of the animals.

Summary Table 2 Data from cattle fed leucaena after 80 days grazing oats and tropical grass pasture

	After 21 days back on leucaena
Leucaena in diet (%)	
Average	45%
Range	29-56
Urine 3,4-DHP concentration (ppm)	
Average	118 ppm
Range	19-302
Urine 2,3-DHP concentration (ppm)	
Average	490 ppm
Range	84-1128

Note - ppm is equal to mg/l