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An investigation into the extent and causes of leucaena toxicity in Queensland

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Abstract

Leucaena leucocephala (leucaena)-grass pastures are productive, profitable & sustainable. However leucaena contains a toxic amino acid called mimosine. The introduction into Australia of the rumen bacterium *Synergistes jonesii* (the leucaena 'bug'), that detoxifies mimosine & its rumen breakdown product DHP, had solved this problem. However, at the break of the 2003 drought, deaths & severe DHP toxicity symptoms in cattle grazing leucaena were reported in herds previously thought protected by the bug. With support from MLA a study was conducted to investigate the extent & causes of leucaena toxicity in Queensland.

A survey of leucaena growers revealed that many had used inappropriate rumen inoculation methods &/or were unaware of the need for on-going bug management. The toxicity status of 385 animals in 44 herds grazing leucaena was tested. Leucaena toxicity was found to be a significant problem. Subclinical 3,4-DHP toxicity was considered to be limiting animal performance in 20% of these herds. A further 32% were found to be excreting high levels of 2,3-DHP. Of particular concern, many of these herds had been inoculated with the bug & were considered protected. Method of bug inoculation was linked to herd protection status & direct drenching with *S. jonesii* culture was most effective. The discovery of partially protected herds with animals excreting large amounts of 2,3-DHP was surprising & highlighted the need for more research into detoxification processes & the ecology of the bacteria involved. Graziers require accurate information about leucaena toxicity & its prevention to enable them to implement effective rumen inoculation & post-inoculation bug management strategies in order to maximize herd productivity.

Executive Summary

Leucaena leucocephala (leucaena) is used throughout the tropics as forage for beef cattle. Leucaena-grass pastures are the most productive, profitable & sustainable improved pastures available for graziers in northern Australia. However, leucaena does contain a toxic amino acid called mimosine. Both mimosine & its ruminal breakdown product DHP (3-hydroxy-4(1H)-pyridone) are toxic to cattle. A specific rumen bacterium, called *Synergistes jonesii*, or the leucaena 'bug', can detoxify DHP & protect cattle from leucaena toxicity. This bacterium was introduced to Australia in 1982 & has spread rapidly within inoculated herds grazing leucaena. Prior to inoculation with the bug, cattle were restricted to eating diets containing less than 30% leucaena to minimize the impact of DHP toxicity. The productivity of cattle grazing leucaena-grass pastures increased dramatically (e.g. liveweight gain increased by 30 to 100%) once the bug was introduced to herds & overcame DHP toxicity. Due to the early success & rapid adoption of the bug, graziers & researchers thought that the leucaena toxicity problem was solved. However, at the break in 2003 drought, a number of graziers in Central Queensland reported cattle deaths (mimosine toxicity) & symptoms of severe DHP toxicity in cattle grazing lush leucaena regrowth. Toxicity occurred in herds previously considered protected by the bug. These events indicated that the bug may not be efficiently protecting cattle & that many herds grazing leucaena might be performing below their potential due to subclinical DHP toxicity.

This project, supported by MLA, was initiated to investigate the extent & causes of leucaena (mimosine & DHP) toxicity in Queensland. There were two components to the study. A postal/telephone survey of all known leucaena growers sought information regarding:

- the level of grazier awareness about leucaena toxicity;
- the prevalence of toxicity symptoms observed by graziers;
- current methods of rumen inoculation & on-going herd management to retain the bug; &
- other cattle management practices that may affect the efficacy of *S. jonesii*.

Concurrently, a herd testing study sought to quantify the prevalence of mimosine/DHP toxicity in a sample of Queensland cattle herds grazing leucaena.

The postal/telephone survey was conducted during May-July 2004. A high rate of participation was achieved with 55% (195 of 356) of graziers responding to the survey. Of these respondents: 171 (88% of 195) were current leucaena growers; 19 (10% of 195) were planning to plant leucaena; & 5 (3% of 195) were not currently growing leucaena & were not interested in planting it in future. The survey found that leucaena-grass pastures were an important (100,000-125,000 ha) & expanding forage resource for the Queensland beef industry, supporting 114,000-155,000 head of cattle annually. Whilst most (73%) graziers thought they understood leucaena toxicity & its prevention:

- 10% of respondents had made no attempt to protect their cattle from toxicity;
- many had used scientifically unproven (risky) methods of rumen inoculation (e.g. acquiring manure & trough water from 'protected' properties) to introduce the bug to their herds;
- many had not followed the recommended DPI&F drenching protocol &/or had not implemented on-going cattle management to ensure viable populations of the bug were maintained in their herds; &
- 30% had inoculated their herd more than once, suggesting that they were not confident that their earlier attempts to introduce & maintain the bug had been successful.

The toxicity status of 385 animals from 44 individually managed herds across Queensland was tested in the summer of 2003/04. Urine & faecal samples were collected from animals grazing leucaena. The concentrations of the toxins mimosine, 3,4-DHP & 2,3-DHP in the urine were measured & the amount of leucaena in the diet of animals determined from the faecal samples. The management practices imposed on each herd were also recorded. The study found:

- the average level of leucaena in the diet of the animals tested was 35% & was considered sufficient to induce excretion of significant amounts of the toxins in the urine of unprotected animals;
- mimosine was present in very low concentrations (average=11 PPM), indicating none of the animals tested were suffering from acute mimosine toxicity;
- only 48% (21 of 44) of the herds tested were completely protected from both 3,4-DHP & 2,3-DHP toxicity;
- 20% (9 of 44) of herds were not protected & were suffering from hidden (subclinical) DHP toxicity. Of concern, 6 of these herds had been inoculated with the bug & the graziers managing these herds had thought they were protected;
- inexplicably, animals in 32% (14 of 44) of tested herds had high urinary concentrations of 2,3-DHP but trace amounts of 3,4-DHP. These herds were classified as partially protected from leucaena toxicity because 2,3-DHP is also toxic; &
- method of rumen inoculation was linked, although the statistical confidence of the relationship was weak, to herd protection status. Data suggested direct drenching with DPI&F *S. jonesii* culture proved most effective in preventing DHP toxicity.

The study found that leucaena toxicity is still occurring in Queensland. These results indicate that the industry (graziers, researchers & extension staff) had generally become complacent about the threat of leucaena toxicity, & that many graziers were not using recommended inoculation protocols & were unaware of the need for proactive management of the *S. jonesii* rumen bug. The herd testing study found that less than half of the herds tested were completely protected from DHP toxicity. Twenty percent were suffering from subclinical (hidden) 3,4-DHP toxicity, while the discovery of partially protected herds with high urine 2,3-DHP levels was surprising, it highlighted shortfalls in our current understanding of how these toxins are broken down in the rumen. A key finding was the need to provide graziers with detailed information about leucaena toxicity & its prevention to enable them to implement effective herd inoculation & post-inoculation bug management strategies.

A conservative estimate of the value of lost production if 20% of the national herd grazing leucaena are suffering from subclinical 3,4-DHP toxicity is >$1.8M/yr. The effect of the additional 32% of herds suffering from 2,3 DHP toxicity is unknown, although 2,3 DHP is a reported appetite suppressant. Therefore prevention of leucaena toxicity will have significant economic benefits to both individual graziers and the northern beef industry. The immediate impact of the project has been to raise the level of awareness about leucaena toxicity amongst 350 leucaena growers through the survey process. The distribution of a summary of the project findings & a 'Leucaena Toxicity Prevention Fact Sheet' to over 215 graziers & extension staff, who participated in the study, will provide graziers with the information they need to implement effective rumen inoculation & herd management strategies. Queensland Department of Primary Industries & Fisheries will continue distributing the 'Leucaena Toxicity Prevention Fact Sheet' generated by the project to all graziers who order *S. jonesii* culture. Following these protocols should enable graziers to limit the occurrence of subclinical DHP toxicity, thereby safeguarding the health & productivity of over 114,000 cattle grazing leucaena pastures each year.

On-going extension activities, such as leucaena field days, training courses & the production of the MLA-funded book '*A Graziers Guide to Leucaena Establishment & Management*' (NBP.224), will ensure that future leucaena growers are well informed about this important production issue. However, urgent applied research is required to improve our scientific understanding of DHP detoxification pathways, the rumen ecology of the bacteria involved in these processes, the mechanisms of transfer/spread of the bacteria within herds & the impact that common herd management practices have on the efficacy of these bacteria. The results of this research will enable graziers to adapt herd management protocols to ensure the long-term protection of cattle from leucaena toxicity & maximize animal production.

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1 Background

1.1 Leucaena toxicity

1.1.1 Leucaena in Queensland

The leguminous shrub *Leucaena leucocephala* (leucaena) is widely used as forage for beef cattle production in tropical agriculture. Leucaena-grass pastures are one of the most productive, profitable and sustainable improved pasture options for northern Australia. At present more than 100,000 ha of commercial leucaena-grass pastures have been established in Central Queensland and there is further potential for rapid adoption of leucaena pastures by the northern Australian beef industry over the next 10 years. Apart from high productivity, reasons for further expansion include: 1) leucaena pastures can ameliorate dryland salinity when planted in recharge areas of priority catchments of the National Action Plan for Salinity & Water Quality in Qld; 2) leucaena is a proven drought mitigation strategy; and 3) the psyllid-resistant *Leucaena* hybrid currently being developed through MLA project NBP.307 will significantly extend the area suitable for establishing leucaena into humid areas and the seasonally cool subtropics.

1.1.2 Reports of leucaena toxicity

At the break of the drought in early 2003, there were a significant number of cases of severe leucaena toxicity, with some animal deaths, in cattle grazing leucaena in Central Queensland (e.g. Mr Greg Coutts, Wandoan). These were apparently due to mimosine toxicity. The symptoms were acute in nature, appearing after 1-2 weeks in animals consuming young fresh leucaena regrowth. Other graziers reported symptoms of 3-hydroxy-4(1H)-pyridone (DHP) toxicity in their herds. Of particular concern was that both forms of leucaena toxicity occurred on properties whose owners had thought that they had satisfactorily inoculated cattle and therefore protected their herds.

1.1.3 What is leucaena toxicity?

Leaves, pods and seed of commercial leucaena cultivars contain significant amounts (4-12% dry matter (DM)) of mimosine, a non-protein amino acid (Jones 1994). Both mimosine and its primary ruminal degradation product DHP (Hegarty *et al*. 1964) are toxic. Mimosine toxicity occurs quickly due to its severe impact on cell division and animals can exhibit symptoms within 3 days of consuming large amounts of leucaena (Vohradsky 1972). Pathological evidence of acute mimosine toxicity include: loss of appetite; ataxia; hair loss; conjunctivitis; ulceration of the tongue, oesophagus and rumen; and congestion, haemorrhaging, and necrosis of the kidneys and liver (Vohradsky 1972; Jones & Megarrity 1986; Prasad & Paliwal 1989). Severe cases of acute mimosine toxicity often result in the death of the animal. However, due to the rapid degradation of mimosine to DHP by plant enzymes released by chewing (Lowry *et al.* 1983) and by a suite of microbes in the rumen (Hegarty *et al.* 1976), mimosine toxicity is rare. DHP toxicity is more common. DHP (free or conjugated with glucuronide) is a potent goitrogen (Hegarty *et al*. 1976) causing hyperplasticity of the thyroid gland, and impeding iodine binding and thyroxine production (Hegarty *et al*. 1979). Clinical toxicity symptoms occur in cattle when leucaena comprises over 30% of dietary DM intake or mimosine over 1% of DM intake (Jones & Hegarty 1984) and the toxic effects appear to be cumulative, as symptoms often emerge after 2-3 months of leucaena consumption (Blunt 1976; Blunt & Jones 1977; Quirk *et al*. 1988). Clinical symptoms include: lethargy; hair loss (especially around the pizzle, tail and rump); lesions on skin; excessive salivation; goitre; cataracts; reduced blood thyroxine concentrations; depressed appetite and growth; poor breeder reproductive performance (embryonic death/abortion); and the production of weak, goitrous calves (Hamilton *et al*. 1971; Holmes 1976; Holmes *et al*. 1981; Jones & Lowry 1984; Jones *et al*. 1989). Subclinical DHP toxicity is not visible but can significantly retard animal growth (by depressing appetite) without manifesting any of the aforementioned clinical symptoms (Jones & Winter 1982; Quirk *et al*. 1988). Leucaena toxicity

has limited animal production from leucaena-grass pastures to such an extent that it prevented adoption of these pastures by graziers, particularly in the wet tropics and under irrigation in the dry tropics (Blunt 1976; Falvey 1976; Holmes 1976, 1979 & 1981).

1.1.4 The leucaena 'bug' - *Synergistes jonesii*

The ruminal bacterium *Synergistes jonesii*, an obligatory anaerobic, gram negative, rod-shaped bacteria (Allison *et al.* 1992), isolated from the rumen contents of goats in Hawaii (Jones 1981), was found to degrade mimosine and DHP to harmless by-products (Jones & Megarrity 1986). Rumen inoculation of cattle in Australia in 1982 with cultured strains of the bacterium successfully protected them against DHP toxicity in experimental trials (Jones & Megarrity 1986). Despite *S. jonesii* being an obligate anaerobe, these studies found that the bacterium was easily and rapidly transmitted between grazing animals (Jones *et al*. 1985b; Quirk *et al*. 1988; Hammond *et al*. 1989; Pratchett *et al*. 1991). The mechanism of passive animal-to-animal transfer was never identified. The rumen bacterium was then made available to commercial cattle producers utilizing leucaena-grass pastures. The Queensland Department of Primary Industries and Fisheries (DPI&F) has developed an *in vitro*, fermentor-based, mixed culture bacterial inoculum, which includes *S. jonesii* (Klieve *et al.* 2002). This rumen inoculum is administered to cattle via an oral drench and effective protection of a herd can be achieved when >10% of animals are inoculated (Klieve *et al*. 2002).

Until these recent reports of animal deaths and toxicity symptoms, many scientists and graziers believed that the problem of mimosine/DHP toxicity was resolved and that, once the bacterium was introduced to a herd, animals were permanently protected provided some simple guidelines were followed.

1.2 Our hypothesis

We hypothesized that cattle, in poor condition at the end of the 2002/03 drought, were gorging themselves on abundant fresh leucaena, which can contain very high mimosine concentrations (up to 12% DM), thereby ingesting large amounts of mimosine. The normal process of mimosine conversion to DHP by plant enzymes and rumen microbes was overwhelmed, resulting in the absorption of large quantities of mimosine into the bloodstream. Animal health deteriorated quickly resulting in sudden death. Post-mortem examination of an animal on the property of Mr Greg Coutts revealed ulceration of the mouth, and congestion and haemorrhaging of the liver and kidneys (Dr Bevan Peters, Wandoan Veterinarian, personal communication), confirming acute mimosine toxicity as distinct from DHP toxicity. Under these extreme environmental conditions, other graziers reported symptoms of severe DHP toxicity (hair loss and excessive salivation) in their herds. Mimosine toxicity, such as reported above, is likely to be rare and therefore not a major issue except that it indicates that the herd was not protected by *S. jonesii* as previously thought.

The lack of protection from DHP toxicity is of serious concern, however, as in normal seasons uninoculated cattle rarely exhibit clinical symptoms of DHP toxicity, which can take 2-3 months to develop (Blunt 1976; Blunt & Jones 1977; Jones & Winter 1982). Thus animals can appear healthy while suffering appetite and growth suppression (i.e., they may be achieving liveweight gains (LWG) of 0.7 kg/day when >1.0 kg/day is feasible). The total economic loss due to under-performance may be significant. This was the case at DPI&F Brian Pastures Research Station, where for 20 years, cattle grazing leucaena performed 'well' without exhibiting symptoms of toxicity. When *S. jonesii* was introduced to the station's herd LWG of steers grazing leucaena was increased from 0.52 to 1.02 kg/hd/d (Quirk *et al*. 1988). Cattle LWG improved from 830 kg/ha/yr to 1442 kg/ha/yr with *S. jonesii* inoculation of cattle grazing irrigated leucaena in the Kimberley (Jones 1994). Prior to inoculation, subclinical DHP toxicity suppressed LWG by >30% in cattle grazing these irrigated leucaena pastures (Jones & Winter 1982).

We hypothesized that many herds grazing leucaena may be adversely affected by subclinical DHP toxicity. Graziers are uncertain of the rumen bacterium status of their herds and some may wrongly believe that their cattle are protected against toxicity. Preliminary enquiries

indicated that, while many graziers had inoculated their herds as recommended, some graziers had not inoculated their cattle or had been using untested and inappropriate inoculation procedures. Furthermore, graziers did not know how cattle should be managed to ensure retention of the bacterium in herds, especially where animals are being continuously bought and sold.

1.3 Development of the research project

It was important that the full extent of mimosine/DHP toxicity within Queensland cattle herds grazing leucaena be determined. If the prevalence of toxicity was as widespread as hypothesised, especially in previously inoculated herds, then a research and education campaign was urgently needed. The results of such studies would lead to more effective rumen inoculation procedures and herd management strategies to ensure viable populations of *S. jonesii* are maintained in cattle herds grazing leucaena. This information would enable graziers to realize the full production and economic benefits of the leucaena-grass production system.

2 Project Objectives

The research project sought to:

1. Determine by telephone/postal survey the level of awareness of leucaena toxicity amongst graziers and to ascertain the methods of rumen inoculation and herd management currently in use.
2. Definitively quantify the prevalence of mimosine/DHP toxicity in a sample of Queensland cattle herds grazing leucaena, by observing and testing animals.

3 Methodology

3.1 Grazier survey methodology

3.1.1 Developing the survey questionnaire

A survey questionnaire (Appendix 1) was carefully constructed in consultation with statisticians to ensure questions were clear and unbiased. The survey aimed to gather the following information from leucaena growers:

<u>The leucaena resource</u>

1) Basic information about each grazier's leucaena resource (area planted, cultivars used, planting date etc)
2) The role leucaena played in their production system and the number of animals grazing leucaena annually (what is leucaena used for e.g. fattening, weaning, drought reserve etc)

<u>Grazier awareness of toxicity and its prevention</u>

1) The level of awareness graziers had about leucaena toxicity; their level of understanding of the issue; and the degree of confidence they had that their toxicity prevention strategies were working.

<u>Information about the prevalence and extent of toxicity</u>

1) The prevalence of clinical toxicity observed by graziers was determined. The proportion of affected animals within the herd and the symptoms observed were recorded.
2) Graziers were asked if they had received reports of post-slaughter abnormalities in their cattle grazing leucaena. Average LWG data were collected, where available, as a measure of animal performance in an effort to detect subclinical leucaena toxicity.

Information regarding rumen inoculation procedures
1) How many graziers had or had not inoculated their cattle?
2) How many had obtained the rumen bug from DPI&F and had followed the recommended inoculation protocol?
3) How many graziers had used other techniques to introduce the bug to their herds?
4) Were graziers actively managing their herds to retain the bug?

Animal management
1) Graziers were asked questions about animal management practices imposed while grazing leucaena to determine if any management practices promoted or decreased the efficiency of protection of cattle from leucaena toxicity by *S. jonesii* (e.g. supplementation strategies, destocking leucaena etc).

Grazier comments and suggestions
1) What factors did graziers suggest either prevented or contributed to leucaena toxicity?

3.1.2 Survey methodology

Researchers at The University of Queensland collated a statewide database of potential leucaena growers drawing on the knowledge of members of The Leucaena Network, seed merchants and regional DPI&F staff. Staff of DPI&F Brian Pastures Research Station, Gayndah, maintain an additional database of leucaena growers who have requested the *S. jonesii* rumen bug since 1995. To maintain client confidentiality, DPI&F mailed surveys to these leucaena growers on behalf of the UQ research team. A total of 356 surveys were posted to potential leucaena growers in April 2004. A covering letter explaining the research project and its objectives accompanied the questionnaire. Covering letters from both The Leucaena Network and DPI&F (the 'gatekeepers' of the databases) endorsing grazier participation in the study were also enclosed in the survey package, along with a form by which graziers could request a summary of the survey findings. Two reply-paid envelopes (one for the survey and one for the summary request form) were provided to the graziers to enable them to return the surveys with minimal inconvenience and to maintain anonymity.

The questionnaire and survey methodology were assessed by the Behavioural and Social Science Ethical Review Committee of The University of Queensland to ensure they complied with provisions stated in the *National Statement on Ethical Conduct in Research Involving Humans*. The committee approved this survey activity (Clearance No: 2004000232).

3.1.3 Statistical analysis

Summary statistics for the responses to the survey were prepared and relationships between respondent's answers to questions were investigated using Chi squared tests (Minitab 12, Minitab Inc, PA, USA).

3.2 Herd testing methodology

3.2.1 Experimental design

The toxicity status of 385 animals from 44 individually managed herds within 6 geographic areas across Queensland (Table 1) was tested during the summer of 2003-04. The following criteria for herd selection were set to ensure cattle were consuming adequate amounts of leucaena (mimosine) to enable the determination of their toxicity status:
1) the area of leucaena under grazing exceeded 40 ha;
2) areas were sampled after rain events that enabled good leucaena growth; and
3) cattle had been grazing leucaena for at least 3 weeks prior to sampling.

The manager of each herd was asked to complete a questionnaire (Appendix 2) that sought information about preceding climatic conditions, animal management, animal performance and the history of herd inoculation with *S. jonesii*. The University of Queensland Animal Ethics Committee approved this research (AEC Number: SLAFS/654/03/MLA).

Table 1: The number of herds sampled from districts throughout Queensland.

District	Number of herds
Far North	1
Clermont/Capella	7
Biloela/Moura/Theodore	15
Injune/Rolleston	3
Gayndah/South Burnett	13
Taroom/Wandoan	5
Total	**44**

3.2.2 Sample collection

Cattle were removed from pasture and yarded immediately prior to sample collection. Paired urine and faeces samples were collected randomly from 2-12 animals in each herd. To preserve the toxins present in the urine, samples were acidified immediately by diluting 20:1 with 32% HCl. Faecal samples were collected directly from the rectum of each animal. The urine and faeces samples were placed on ice in the field and then stored at <4ºC.

3.2.3 Sample preparation

Urine samples were diluted 1:4 with 0.1M HCl and then filtered, purified and hydrolysed prior to high performance liquid chromatography (HPLC) analysis. Particulate contaminants and non-polar urinary metabolites were removed from the samples to protect the HPLC column and improve the accuracy of the analysis. Samples were passed through Minisart® 0.45 μm cellulose acetate filters (Sartorius AG, Goettingen, Germany) followed by reverse phase solid phase extraction on Maxi-Clean™ 300 mg C18 cartridges (Alltech Associates Inc, Deerfield, USA) at a flow rate of 3 ml/min. The urine samples were then hydrolysed to release DHP that may be conjugated with glucuronides, as the conjugated form may account for 30-70% of plasma (Hegarty *et al*. 1979) and 35% of urinary DHP (Hegarty *et al*. 1964). Purified urine was diluted 1:1 with 32% HCl and placed in a 90ºC water bath for 60 min. Faecal samples were oven dried at 65ºC, ground through a hammer mill with a 1 mm sieve and then pulverized to a homogenous fine powder in a roller mill for 72 hours.

3.2.4 Sample analysis

The urinary concentrations of mimosine, 3,4-DHP and 2,3-DHP were determined using a modification of the HPLC procedure of Tangendjaja and Wills (1980). A 0.2 ml aliquot of hydrolysed urine was passed through a Luna 5μ C18(2) 100 Å 150 mm × 4.6 mm column (Phenomenex Inc, Torrance, USA) attached to a Agilent 1100 Series HPLC machine (Agilent Technologies Inc, Palo Alto, USA) in a 0.05M ammonium acetate (pH 2.25) mobile phase at a flow rate of 1 ml/min. Concentrations of toxins were measured using a UV detector at λ = 270 nm. Standard mimosine and 2,3-DHP (Sigma-Aldrich Pty Ltd, St Louis, USA) were used to calibrate the HPLC. As 3,4-DHP was not commercially available, standard material was synthesized from mimosine using the procedure of Hart *et al*. (1977). The standard 3,4-DHP produced was confirmed using liquid chromatography/mass spectrometry (LCMS). The identity of the compounds present in the urine samples, that had the same elution times as the standards, was also validated by LCMS. The proportion of C-3 (leucaena) and C-4 (tropical grass) plant material in the animals' diet was determined by delta carbon (δC^{13}) analysis of the faecal powder (Jones *et al*. 1979) using dynamic flash combustion (Carlo Erba 1110 Elemental Analyser) and

continuous flow mass spectrometry (VG Isochrom-EA Mass Spectrometer). Leucaena was assumed to be the sole C-3 species grazed in leucaena-grass pastures during summer.

3.2.5 Statistical analysis

Relationships between diet composition and urinary toxin concentration were investigated by normal regression analysis (Microsoft Excel 2000). Raw and log-transformed toxin data were compared to the herd management questionnaire data using pivot tables, 1-way ANOVA (Minitab 12) and logistic regression (SAS version 8.02, SAS Institute Inc, CN, USA). Any significant relationships identified from the logistic regression were further tested by Chi squared and Fisher's exact tests (SAS).

4 Results and Discussion

4.1 Postal survey results

A very high rate of participation was achieved with 55% (195 of 356) of graziers responding to the survey. Of these respondents: 171 (88% of 195) were current leucaena growers; 19 (10% of 195) were planning to plant leucaena in the near future and were very interested in the findings of the research project; and 5 (3% of 195) were not currently growing leucaena and were not interested in planting it in future.

The raw data of the survey responses are given in Appendix 3 and are briefly summarized below.

4.1.1 The leucaena resource

The total area of leucaena planted by respondents (as at July 2004) was 47,600 ha. The area planted by individual graziers ranged from 2 to 2,430 ha, while the median and mean (± SE) area planted by each grazier was 121 ha and 280 ± 33 ha respectively. Assuming the survey respondents are representative of the broader leucaena-growing community (thought to be at least 400 graziers), the total area of leucaena currently planted in northern Australia is estimated to be approx. 112,000 ± 13,200 ha. The total number of cattle reported by respondents to be grazing leucaena pastures each year was approximately 55,300 hd, with an average number of 337 ± 52 hd/grazier. The total national herd grazing leucaena each year, extrapolated to account for over 400 graziers, would be 134,800 ± 20,800 hd/yr. Respondents indicated that all three commercial varieties currently available (Peru, Cunningham and Tarramba) were contributing significantly to the forage resource. The number of graziers planting leucaena has doubled in the last 5-10 years and is increasing rapidly (Figure 1). Importantly, 67% (114 of 170) of respondents had continued to expand their leucaena plantings over the last 5 years. Graziers were using leucaena pastures predominantly to fatten and finish steers/bullocks, although backgrounding and weaning cattle onto leucaena pastures was also popular.

4.1.2 Grazier awareness of toxicity and its prevention

Most graziers (73%) thought they understood leucaena toxicity and its prevention, while 25% were aware of it but felt they knew little about it, and 2% did not know that leucaena could be toxic. The level of confidence respondents had in the success of their rumen inoculation and herd management procedures mirrored this finding, with 68% sure that their herds were protected, with 29% uncertain and 3% believing their efforts had failed.

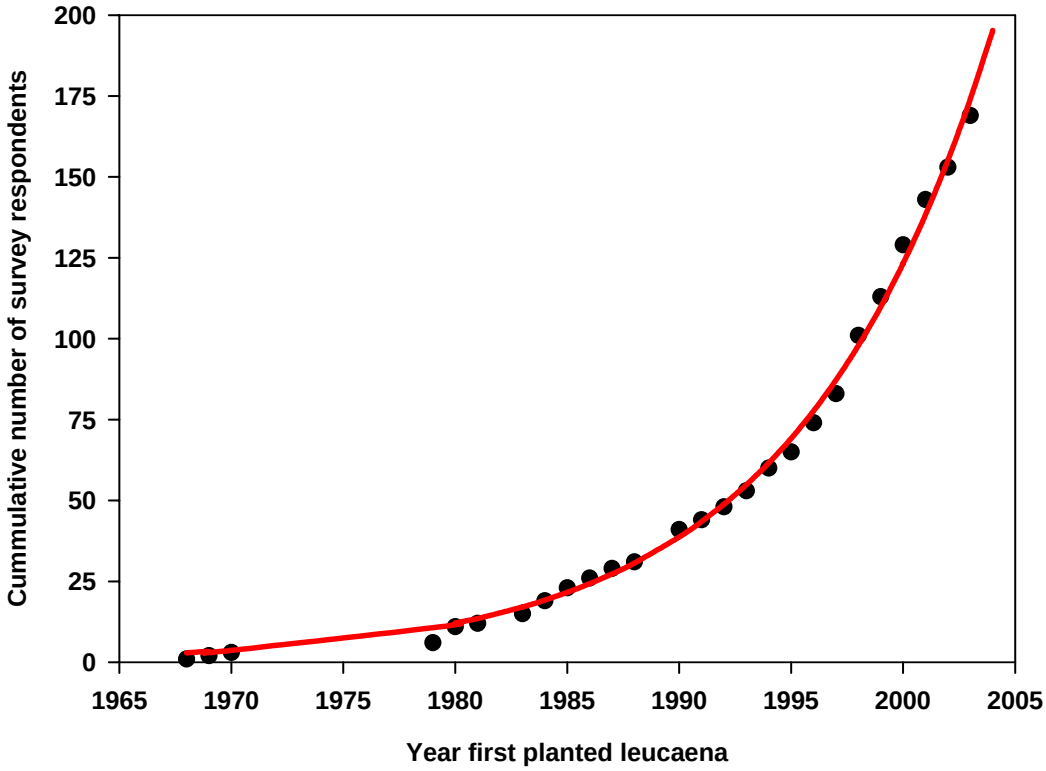

Figure 1: The number of graziers planting leucaena-grass pastures is growing exponentially, as shown by the date survey respondents first planted leucaena.

4.1.3 The prevalence and extent of toxicity

Many graziers (43%) reported they had observed symptoms of leucaena toxicity in their herds. The most common symptoms reported were hair loss and poor performance. Fifteen graziers (9%) had observed unexplained cattle deaths in herds grazing leucaena. Most (93%) reported that symptoms occurred rarely or infrequently. The average (± SE) proportion of animals in affected herds that exhibited symptoms was 17.5 ± 3.8%. Very few graziers (4%) reported post-slaughter abnormalities, such as liver damage, enlarged thyroid glands or lesions in the digestive tract, which are associated with cattle suffering acute mimosine and/or prolonged DHP toxicity.

Eighty-two (48% of 171) graziers weighed their cattle and all reported that their cattle achieved >0.5 kg/hd/d, while 52 (63% of 82) observed LWG ≥1.0 kg/hd/d. Reported animal LWG from leucaena-grass pastures averaged (± SE) 1.05 ± 0.04 kg/hd/d.

4.1.4 Rumen inoculation procedures

Many graziers (63%) had obtained the rumen bug from DPI&F. Most had followed the recommended protocol of drenching >10% of their cattle with 100 ml of inoculum/hd, although 31 graziers (19%) had not. Many graziers (30%) had inoculated their cattle more than once. Of those that had not inoculated directly with DPI&F bug, many had tried to introduce the bug to their herd by transferring trough water (19%), manure (9%) or by borrowing animals thought to have the bug (19%). Surprisingly, 17 graziers (10%) had made no effort to obtain the bug and prevent leucaena toxicity. Only 57% of graziers tried to retain the bug in their herd by maintaining carrier animals on leucaena and mixing them with new animals introduced to leucaena. The remainder implemented no on-going herd management strategy to retain the bug.

4.1.5 Animal management

Graziers were asked whether or not they provided supplements to their cattle while grazing leucaena. Many (58%) did not. Of those that did provide supplements, urea, molasses, protein meal and grain were popular, & were provided predominantly (73%) in winter or drought periods when leucaena forage was in short supply. The remainder provided supplements year-round (predominantly bentonite, mineral licks and water-medicated urea). Approximately half the respondents spelled their leucaena paddocks for a period of greater than 4 weeks each year.

4.1.6 Relationships between answers to the survey

Statistical analyses were conducted to test the following relationships:

1. Did the graziers' perceived level of knowledge about toxicity translate to the implementation of recommended rumen inoculation and herd management protocols? This question sought to test whether graziers had a correct understanding of the prevention of leucaena toxicity, as manifested by following recommended herd management protocols. A Chi squared test (P=0.005) revealed that more graziers who thought they understood leucaena toxicity had not followed recommended management protocols than expected, while more graziers who felt they knew little about leucaena toxicity had followed recommended herd management protocols than expected. This indicated that many graziers who thought they understood leucaena toxicity and its prevention, actually did not have good knowledge of recommended prevention strategies, while those who felt they knew little about toxicity were more likely to seek and follow recommended inoculation and herd management protocols. This finding highlighted the need for an extension program to provide all graziers with accurate, detailed information about current recommended inoculation and herd management strategies to prevent leucaena toxicity.
2. Did the graziers' perceived level of knowledge match their confidence that their management strategy had prevented toxicity? The study found there was no significant relationship between graziers' perceived level of knowledge and their confidence that they had successfully protected their herds from toxicity. This indicated that many graziers remained uncertain of the protection status of their herds regardless of how much they thought they knew about leucaena toxicity.
3. Did the graziers' confidence that their management strategy had prevented toxicity translate into the implementation of recommended rumen inoculation and herd management protocols? The study revealed there was no significant relationship between the respondent's answers to these questions.
4. Was there a relationship between graziers' perceived level of knowledge about toxicity and whether they planted leucaena prior to or after 2000? And was there a relationship between whether graziers implemented recommended herd management strategies to prevent toxicity and whether they planted leucaena prior to or after 2000? The study found there was no relationship between the date graziers first planted leucaena, their perceived level of knowledge, or whether or not the grazier had implemented recommended rumen inoculation and post-inoculation herd management protocols. These results suggest that the extension effort to make graziers aware of recommended leucaena toxicity prevention protocols has had the same level of success (40-45% adoption) with recent leucaena growers (first planting post 2000) as with more experienced leucaena growers (first planting pre 2000).

4.1.7 Limitations of the survey questionnaire

The most important shortcoming in the design of the survey questionnaire was the failure to ask graziers to provide dates when they had observed symptoms of leucaena toxicity in their herds (Appendix 1, Questions 11 and 12). This information could have been matched to inoculation date data, enabling the research team to determine if graziers had inoculated their herds in response to observing toxicity symptoms, or if clinical toxicity had occurred or re-occurred after rumen inoculation with the bug. This finding would have provided valuable information regarding

the success with which grazier rumen inoculation procedures and post-inoculation herd management had protected their herds from leucaena toxicity.

4.2 Herd testing results

4.2.1 Diet composition

Delta carbon analysis of the faecal samples indicated that the average diet of the cattle tested comprised 35 ± 0.7% leucaena, while one animal consumed a diet of 79% leucaena prior to sampling. Average herd diet composition ranged from 7% leucaena to 59% (Tables 2 & 3; Appendix 4). Similarly, other studies have observed diet selection of 30-60% leucaena in cattle grazing leucaena-grass pastures during summer/autumn in southeast Queensland (Jones & Jones 1984; Jones *et al*. 1989; Galgal 2002). Generally the composition of the diet of animals within herds in the present study was consistent and is probably the result of group grazing behaviour that has been observed in cattle grazing leucaena-grass pastures (Galgal 2002). With the exception of 3 herds, where cattle were eating less than 15% leucaena in their diet, all herds were consuming significant amounts of leucaena and were therefore ingesting significant quantities of mimosine. Thus the levels of leucaena intake observed in this study were considered to be sufficient to induce the excretion of significant amounts of the toxins in the urine of unprotected animals.

4.2.2 Concentrations of toxins in urine

The HPLC methodology developed in this study definitively measured the concentration of mimosine, 3,4-DHP and 2,3-DHP. Elution times of 2.05, 3.11 and 8.74 minutes were observed for mimosine, 3,4-DHP and 2,3-DHP standards respectively (Figure 2). Recovery tests conducted in urine samples indicated that these elution times were consistent (data not presented). The compounds eluting at these times from a subset of test urine samples were found to be mimosine, 3,4-DHP and 2,3-DHP by LCMS analysis (data not presented).

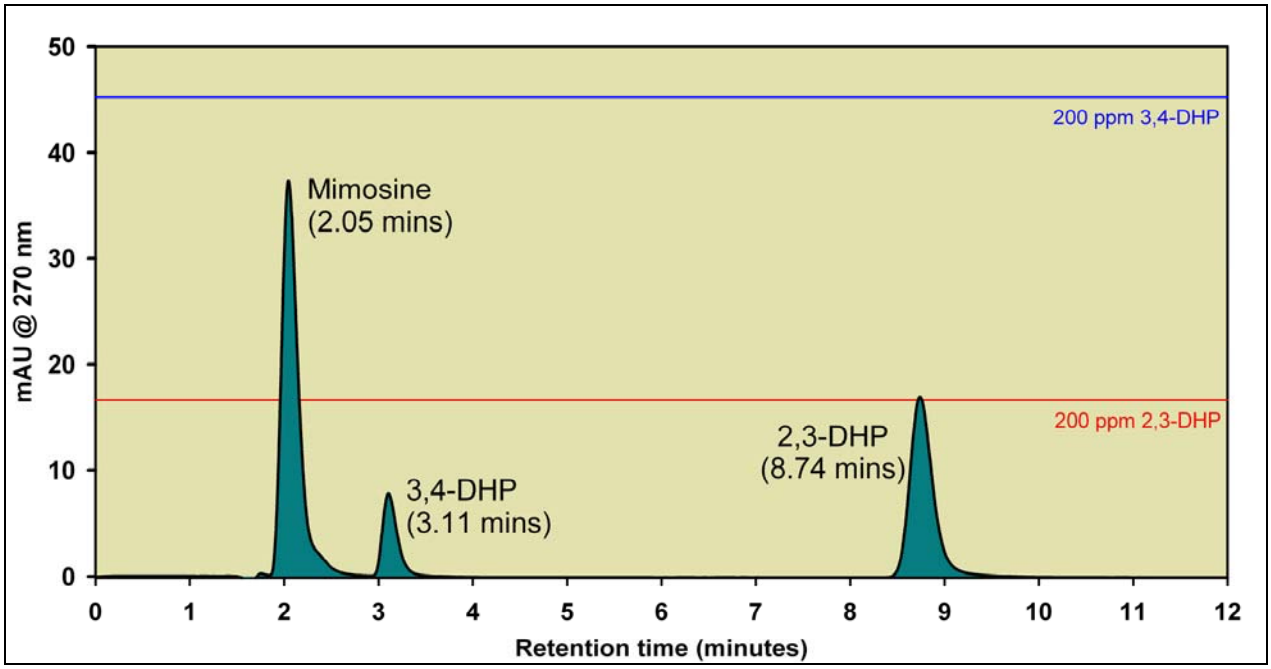

Figure 2: HPLC chromatogram of mimosine, 3,4-DHP and 2,3-DHP standards.

Table 2: Summary statistics for the proportion (%) of leucaena in the diet and concentrations (μg/ml) of mimosine, 3,4-DHP and 2,3-DHP in the urine of animals tested in herds classified as protected from leucaena toxicity.

	Herd No.	n*	Leucaena in diet (%)			Mimosine (μg/ml)			3,4-DHP (μg/ml)			2,3-DHP (μg/ml)		
			Mean ± SE	Median	Range	Mean ± SE	Median	Range	Mean ± SE	Median	Range	Mean ± SE	Median	Range
PROTECTED	1	10	40.4 ± 2.9	39.3	31-57	12.4 ± 0.4	12.5	10 - 14	7.9 ± 0.9	8.4	3 - 13	6.7 ± 3.2	nd	nd - 25
	2	11	32.4 ± 1.5	33.3	24 - 41	18.9 ± 1.6	21.5	12 - 27	5.2 ± 1.3	4.3	nd - 12	nd ± nd	nd	nd
	3	12	25.9 ± 1.7	24.3	18 - 35	13.6 ± 0.1	14.0	13 - 15	11.2 ± 2.4	8.4	nd - 32	nd ± nd	nd	nd
	5	11	24.5 ± 2.0	24.0	17 - 37	14.3 ± 1.6	11.9	9 - 23	7.9 ± 1.5	9.0	nd - 17	14.0 ± 6.7	nd	nd - 70
	6	3	13.3 ± 1.0	14.0	11 - 15	18.3 ± 3.3	21.4	12 - 22	13.4 ± 0.5	13.0	13 - 14	18.1 ± 9.3	23.6	nd - 31
	12	8	50.2 ± 1.4	50.5	45 - 55	12.6 ± 0.2	12.8	12 - 14	7.8 ± 1.8	6.9	nd - 16	8.5 ± 4.2	nd	nd - 28
	14	10	51.0 ± 3.3	52.6	37 - 65	11.0 ± 0.3	11.3	10 - 12	13.7 ± 3.0	12.1	3 - 34	8.7 ± 6.3	nd	nd - 63
	18	10	54.8 ± 2.6	55.4	41 - 67	10.5 ± 0.2	10.4	10 - 11	9.5 ± 1.6	8.7	4 - 19	10.4 ± 2.4	12.5	nd - 20
	23	10	38.9 ± 1.6	40.2	27 - 47	8.6 ± 0.3	8.4	7 - 10	21.1 ± 5.3	12.1	8 - 52	8.6 ± 1.6	9.9	nd - 14
	26	7	46.6 ± 8.7	48.0	41 - 50	6.0 ± 1.0	6.9	nd^ - 8.2	3.5 ± 0.9	3.1	nd - 7	14.4 ± 5.0	9.6	nd - 32
	29	8	27.8 ± 3.5	25.3	17 - 48	8.5 ± 0.3	8.3	8 - 10	10.2 ± 1.8	11.7	3 - 16	34.6 ± 8.2	36.3	7 - 79
	30	4	28.1 ± 6.9	30.1	11 - 41	8.7 ± 1.6	10.1	4 - 11	41.7 ± 18.0	31.3	13 - 92	10.7 ± 7.0	6.6	nd - 30
	31	10	20.3 ± 1.1	19.3	17 - 29	8.2 ± 0.5	8.2	6 - 10	9.6 ± 1.0	8.9	5 - 15	nd ± nd	nd	nd
	33	10	49.0 ± 1.9	48.4	39 - 61	7.8 ± 0.2	7.7	7 - 9	8.9 ± 1.1	7.5	5 - 16	19.2 ± 2.7	18.0	8 - 36
	34	10	34.2 ± 2.4	33.4	25 - 52	5.8 ± 0.8	6.5	nd - 9	3.3 ± 0.8	2.3	nd - 7	4.3 ± 2.2	nd	nd - 16
	37	6	42.4 ± 2.3	41.1	36 - 50	9.9 ± 0.8	10.6	6 - 11	8.5 ± 2.5	6.1	4 - 20	38.0 ± 22.2	17.4	nd - 142
	38	3	46.7 ± 2.6	44.4	44 - 52	11.6 ± 0.2	11.6	11 - 12	5.3 ± 1.0	5.8	3 - 7	25.6 ± 6.9	19.7	18 - 39
	41	4	48.3 ± 0.9	48.5	46 - 50	9.0 ± 0.1	8.9	9 - 9	11.1 ± 2.9	9.8	6 - 19	6.3 ± 3.7	5.7	nd - 14
	42	10	23.5 ± 0.8	22.5	20 - 27	6.8 ± 0.2	6.7	6 - 8	33.8 ± 9.3	23.1	6 - 98	28.2 ± 9.2	15.5	nd - 84
	43	4	22.4 ± 1.3	22.5	19 - 25	6.8 ± 2.8	7.7	nd - 12	22.5 ± 6.7	25.7	4 - 35	16.4 ± 6.2	18.0	nd - 30
	44	11	16.1 ± 1.2	17.1	9 - 24	9.9 ± 0.1	9.9	10 - 10	16.8 ± 4.2	12.8	7 - 57	11.8 ± 4.9	11.1	nd - 55

*n = number of head tested ^nd = not detected

Table 3: Summary statistics for the proportion (%) of leucaena in diet and concentrations (μg/ml) of mimosine, 3,4-DHP and 2,3-DHP in the urine of animals tested in herds classified as partially protected and not protected from leucaena toxicity.

	Herd No.	n*	Leucaena in diet (%)			Mimosine (μg/ml)			3,4-DHP (μg/ml)			2,3-DHP (μg/ml)		
			Mean ± SE	Median	Range	Mean ± SE	Median	Range	Mean ± SE	Median	Range	Mean ± SE	Median	Range
PARTIALLY PROTECTED	4	10	17.7 ± 0.5	17.7	15 - 21	12.9 ± 0.1	13.0	12 - 14	13.3 ± 3.8	8.9	4 - 46	138.8 ± 66.5	46.2	17 - 703
	7	11	58.5 ± 2.6	57.7	50 - 79	11.5 ± 0.2	11.4	11 - 13	48.4 ± 12.2	32.2	7 - 130	110.5 ± 35.3	71.1	11 - 389
	8	7	6.9 ± 0.8	7.0	5 - 9	9.2 ± 0.3	9.0	8 - 11	61.4 ± 22.5	28.8	16 - 168	180.4 ± 85.4	72.6	47 - 679
	15	11	58.2 ± 3.0	60.0	41 - 72	12.8 ± 0.7	12.4	10 - 18	47.4 ± 13.0	30.3	12 - 157	460.8 ± 168.4	238.5	nd - 1809
	16	8	36.6 ± 2.3	35.9	27 - 46	7.3 ± 0.3	7.2	6 - 9	6.6 ± 1.4	7.8	1 - 13	190.6 ± 62.2	128.7	nd - 511
	17	10	32.9 ± 1.8	34.9	24 - 40	11.4 ± 0.3	11.1	10 - 13	16.6 ± 3.6	12.9	4 - 39	633.1 ± 189.7	363.7	26 - 1573
	19	7	45.1 ± 1.6	45.9	39 - 49	9.0 ± 1.5	10.0	nd - 12	15.0 ± 3.5	14.6	nd - 26	420.3 ± 127.5	278.4	29 - 995
	20	12	27.0 ± 2.0	25.4	18 - 42	7.3 ± 0.2	7.2	6 - 8	19.4 ± 7.0	11.7	1 - 92	129.8 ± 66.5	37.2	5 - 825
	21	10	43.5 ± 3.2	42.5	32 - 58	10.6 ± 0.2	10.4	10 - 12	11.8 ± 3.0	8.5	4 - 32	401.8 ± 118.6	313.4	48 - 1330
	22	12	35.0 ± 2.0	33.2	23 - 51	9.6 ± 0.2	9.5	9 - 11	9.0 ± 2.3	4.7	3 - 27	147.6 ± 66.5	47.5	nd - 781
	24	11	24.1 ± 2.5	22.9	18 - 33	9.5 ± 0.1	9.5	9 - 10	10.9 ± 3.5	7.0	3 - 40	68.9 ± 21.6	31.7	nd - 227
	25	10	37.5 ± 2.6	37.0	28 - 54	14.4 ± 1.4	13.0	11 - 27	5.3 ± 1.0	5.6	nd - 12	84.7 ± 37.5	40.8	10 - 403
	27	12	33.9 ± 2.2	33.2	22 - 51	6.8 ± 0.6	7.4	nd - 8	14.0 ± 2.7	11.4	nd - 29	159.1 ± 45.3	144.5	8 - 451
	28	10	24.4 ± 2.6	23.1	9 - 38	13.2 ± 0.4	13.0	12 - 16	14.5 ± 4.9	8.0	2 - 47	275.3 ± 123.1	108.2	18 - 1266
NOT PROTECTED	9	8	45.8 ± 1.6	44.5	42 - 56	9.2 ± 0.3	9.0	6 - 11	359.6 ± 108.6	289.6	75 - 1001	39.3 ± 14.4	25.0	15 - 138
	10	10	52.4 ± 2.5	52.5	41 - 66	9.1 ± 0.2	8.9	8 - 10	107.3 ± 19.9	106.7	23 - 223	23.7 ± 4.0	26.8	nd - 43
	11	9	37.6 ± 1.5	37.3	31 - 47	12.4 ± 0.7	12.9	9 - 15	604.0 ± 89.2	594.3	128 - 1041	273.7 ± 78.3	212.9	34 - 694
	13	5	36.3 ± 2.0	36.8	30 - 42	13.2 ± 0.1	13.2	13 - 14	522.5 ± 88.8	585.1	251 - 713	178.7 ± 20.4	194.9	126 - 232
	32	10	13.9 ± 1.9	12.3	5 - 28	14.7 ± 0.5	15.3	12 - 16	133.4 ± 25.1	121.0	28 - 227	61.6 ± 14.2	44.0	16 - 162
	35	10	30.0 ± 1.5	31.2	21 - 36	11.4 ± 0.1	11.4	11 - 12	265.4 ± 26.1	259.5	158 - 406	675.4 ± 114.2	673.1	177 - 1357
	36	10	47.6 ± 1.3	46.8	44 - 57	13.7 ± 0.3	13.9	12 - 15	788.5 ± 207.7	564.0	113 - 1999	287.9 ± 70.0	191.5	35 - 603
	39	8	40.0 ± 1.7	40.5	30 - 46	10.7 ± 0.2	10.6	10 - 11	166.4 ± 57.5	114.0	23 - 478	43.4 ± 8.6	40.9	15 - 75
	40	2	31.4 ± 2.0	31.4	29 - 33	12.2 ± 0.3	12.2	12 - 12	511.7 ± 4.6	511.7	507 - 516	58.5 ± 21.8	58.5	37 - 80

* n = number of head tested ^ nd = not detected

4.2.2.1 Mimosine

Mimosine was present only in trace concentrations (range 0-27 μg/ml; mean = 10.7 ± 0.2 μg/ml) in the urine of all of the animals tested (Tables 2 & 3; Appendix 4), indicating no animals were suffering acute mimosine toxicity. This finding supported observations that no animals in the study were exhibiting symptoms of mimosine toxicity. Acute mimosine toxicity occurs rarely and is induced by a unique set of circumstances. Mimosine toxicity has occurred when hungry cattle gorged themselves on lush leucaena shoots, which contain very high concentrations (5-10% DM) of mimosine, as a sole feed. The resultant high level of mimosine intake overwhelmed the rumen bacterial populations (including *S. jonesii*) that normally convert mimosine to DHP. Large amounts of mimosine were rapidly absorbed into the bloodstream producing fatal damage to internal organs. These unique environmental conditions were not encountered in the present study. Most cattle were consuming a balanced diet of 20-50% leucaena and the data suggest that endogenous plant enzymes (Lowry *et al.* 1983) and rumen bacteria (Hegarty *et al.* 1976) were efficiently degrading mimosine to 3,4-DHP.

4.2.2.2 DHP

4.2.2.2.1 Threshold concentrations for toxicity

A threshold urine concentration for both 3,4-DHP and 2,3-DHP toxicity was determined following a comprehensive review of published experimental results. Earlier studies of leucaena toxicity in cattle have reported urinary concentrations of total DHP (2,3-DHP, 3,4-DHP) & mimosine in 'protected' animals consuming leucaena that were undetectable (Quirk *et al*. 1988), ≤50 μg/ml (Jones and Megarrity 1986; Hammond *et al*. 1989) or ≤100 μg/ml (Raurela & Jones 1985; Pratchett *et al.* 1991). On the other hand, these studies reported very high urinary concentrations (2,800 to 7,800 μg/ml) of total DHP & mimosine in cattle suffering clinical DHP toxicity (Quirk *et al*. 1988; Pratchett *et al*. 1991). However, one study recorded a much lower concentration of 3,4-DHP (146 μg/ml) in the urine of a beast suffering from DHP toxicity (Hammond *et al*. 1989). The very high urine concentrations of 'DHP' in animals suffering severe clinical toxicity reported in this earlier work may be the result of using simple colorimetric analytical procedures that:

1) did not differentiate between 3,4-DHP, 2,3-DHP and mimosine but estimated a combined total DHP and mimosine concentration;
2) were not specific for mimosine and the DHP isomers. For example, the simple colorimetric assay uses $FeCl_3$ to react with the pyridine ring structure of mimosine and DHP to produce a purple (3,4-DHP) or blue (2,3-DHP) coloured complex. Other urinary compounds with similar ring structures, such as phenolics (Megarrity 1981), can also react with the $FeCl_3$ reagent and result in the overestimation of DHP; and
3) employed limited sample preparation to remove contaminant compounds present in urine samples that may have interfered in the accurate measurement of the toxins.

There is very little published data linking urine concentrations of the toxins with the degree of toxicity (e.g. suppression of LWG) suffered by the animals. However, the excretion of DHP is a definitive indicator of whether animals are protected from leucaena toxicity or not. The research team decided that a urinary concentration of 200 μg/ml was a conservative toxicity threshold for both DHP isomers, considering the highly accurate HPLC analysis employed in the current study and that none of the animals tested exhibited symptoms of DHP toxicity. A herd was considered not protected if more than one animal tested excreted either DHP isomer at concentrations exceeding these thresholds. A single animal in the study represented 10-30% of the grazier's herd, a significant proportion of the herd to be under performing due to DHP toxicity.

4.2.2.2.2 Protected herds

Using this criterion, 21 (48% of 44) herds were completely protected from DHP toxicity as all the animals in these herds had 3,4-DHP and 2,3-DHP concentrations of <200 μg/ml in their urine (Table 2 & Appendix 4). An example of the HPLC chromatogram of urine from a protected animal is presented in Figure 3. These results suggest that the rumen microbial detoxification pathway of converting mimosine to 3,4-DHP (by *S. jonesii* and a suit of other rumen bacteria), 3,4-DHP to

2,3-DHP (by *S. jonesii* only) and then 2,3-DHP to harmless by-products (by *S. jonesii* and other bacteria) was working efficiently in these animals.

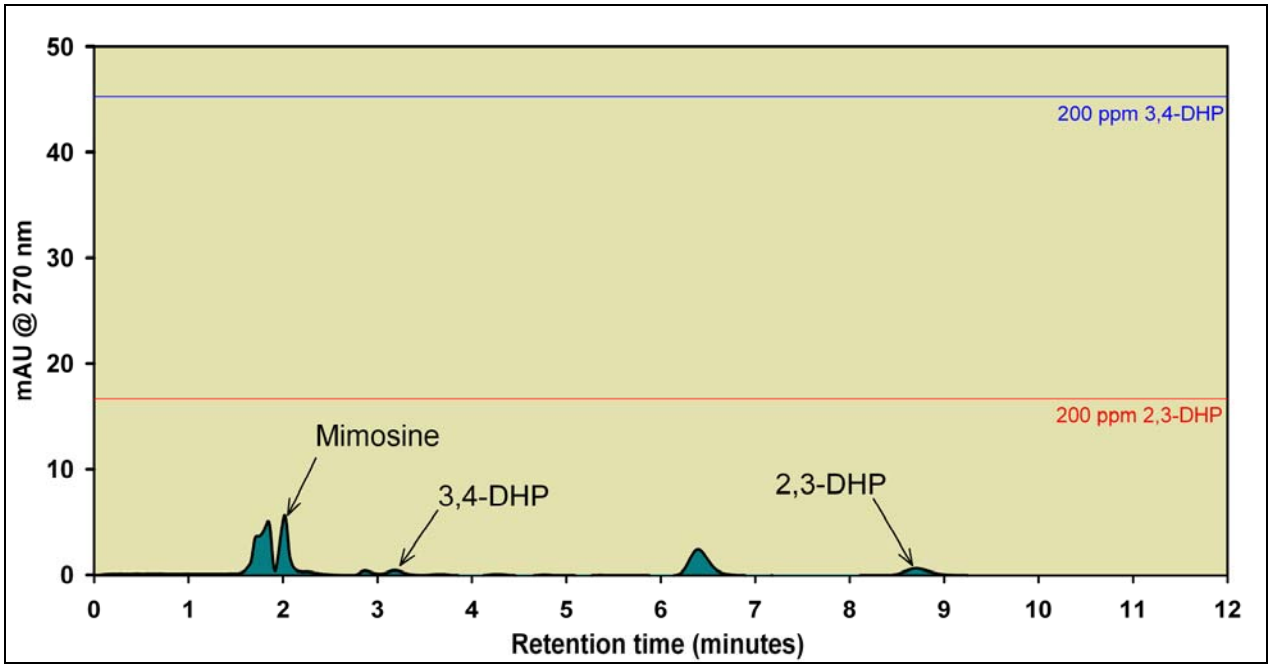

Figure 3: HPLC chromatogram of urine from a protected animal excreting trace amounts of 3,4-DHP & 2,3-DHP.

4.2.2.2.3 Unprotected herds

Nine herds (20% of 44) were not protected by *S. jonesii* and were found to be experiencing subclinical 3,4-DHP toxicity (Table 3 & Appendix 4). This occurred despite 6 of these herds being inoculated with *S. jonesii* (2 had been drenched with DPI&F culture and 4 had been mixed with animals that were 'protected' by the bug). Surprisingly, only 5 of these unprotected herds were suffering 'typical' 3,4-DHP toxicity, which is characterized by animals excreting urine containing high concentrations of 3,4-DHP only (Figure 4). Under circumstances of 'typical' 3,4-DHP toxicity, we assume that *S. jonesii* is not present in the rumen, therefore there is no conversion of 3,4-DHP to 2,3-DHP, and 3,4-DHP accumulates and is excreted in the urine of these animals. Animals in the 4 remaining herds were excreting high concentrations of both 3,4-DHP and 2,3-DHP in their urine (Figure 5). The excretion of both 3,4-DHP and 2,3-DHP indicated that *S. jonesii* was present, but was not effective in rapidly converting 3,4-DHP to 2,3-DHP as is usually the case. Furthermore, *S. jonesii* and other 2,3-DHP degrading bacteria were not effectively breaking down the 2,3-DHP produced.

Unfortunately, only 3 of the graziers managing unprotected herds had recorded reliable LWG data for the animals tested. The cattle in herds No.39 and No.40 had LWG of 0.34 kg/hd/d and 0.04 kg/hd/d respectively, over the 30 days prior to testing which was much lower than the 0.73 kg/hd/d LWG recorded for a protected herd on the same property grazing similar pastures at the same time. Cattle in herd No.35 had LWG over the 60 days preceding sampling of 0.4 kg/hd/d, which was also lower than expected from a top-quality leucaena pasture. These LWG data confirmed that subclinical toxicity was limiting animal performance in some of the unprotected herds.

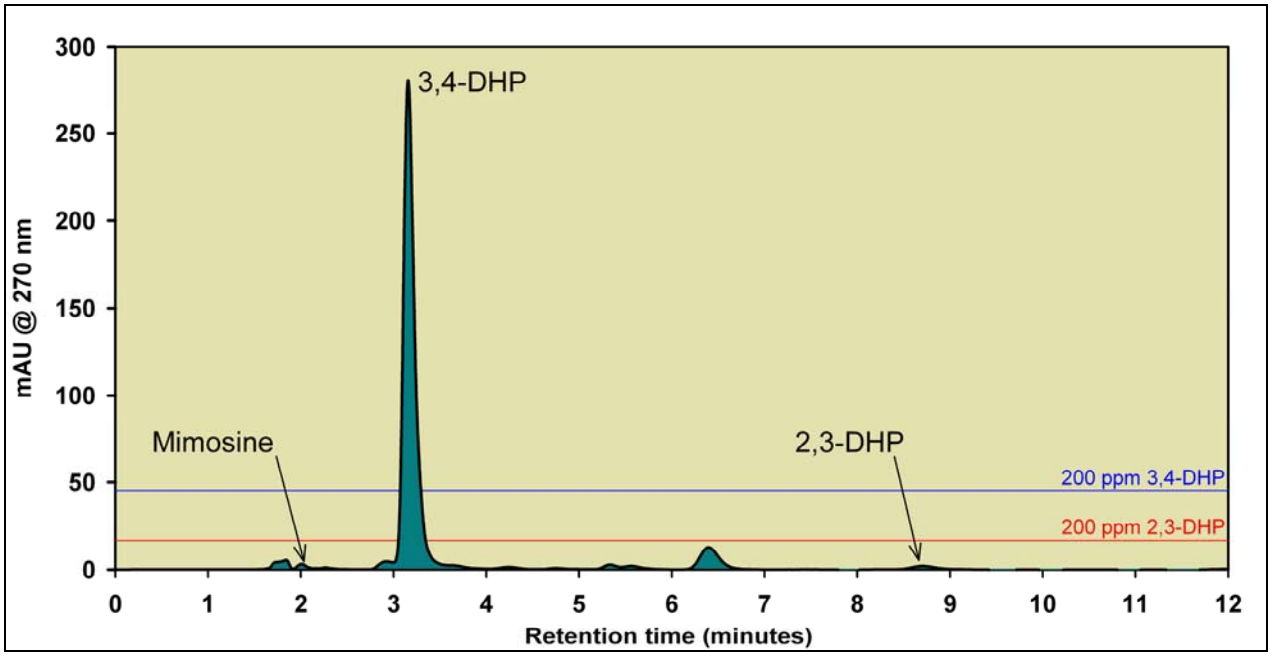

Figure 4: HPLC chromatogram of urine from an animal suffering 'typical' 3,4-DHP toxicity.

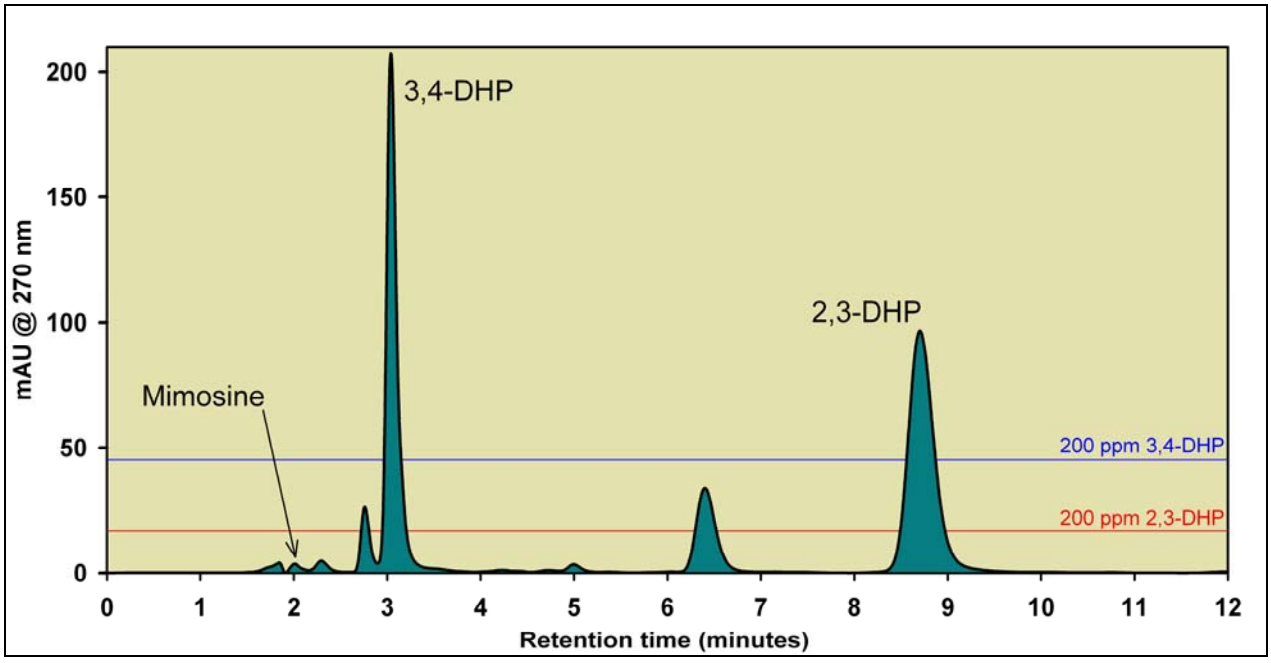

Figure 5: HPLC chromatogram of urine from an animal suffering from 3,4-DHP toxicity but excreting high concentrations of <u>both</u> 3,4-DHP & 2,3-DHP.

4.2.2.2.4 Partially protected herds

Unexpectedly, animals in 14 (32% of 44) herds were excreting low 3,4-DHP concentrations but high 2,3-DHP concentrations (>200 μg/ml) in their urine (Table 3 & Appendix 4). These herds were considered only partially protected because 2,3-DHP is a goitrogen (Lee *et al*. 1980) and appetite suppressant (McSweeney *et al*. 1984). An example of the HPLC chromatogram of urine

from a partially protected animal is presented in Figure 6. These results indicate that *S. jonesii* was present and was converting 3,4-DHP to 2,3-DHP in the rumen. However, *S. jonesii* and other rumen bacteria were not efficiently degrading 2,3-DHP to harmless by-products and the excess 2,3-DHP was being absorbed into the bloodstream and excreted in the urine.

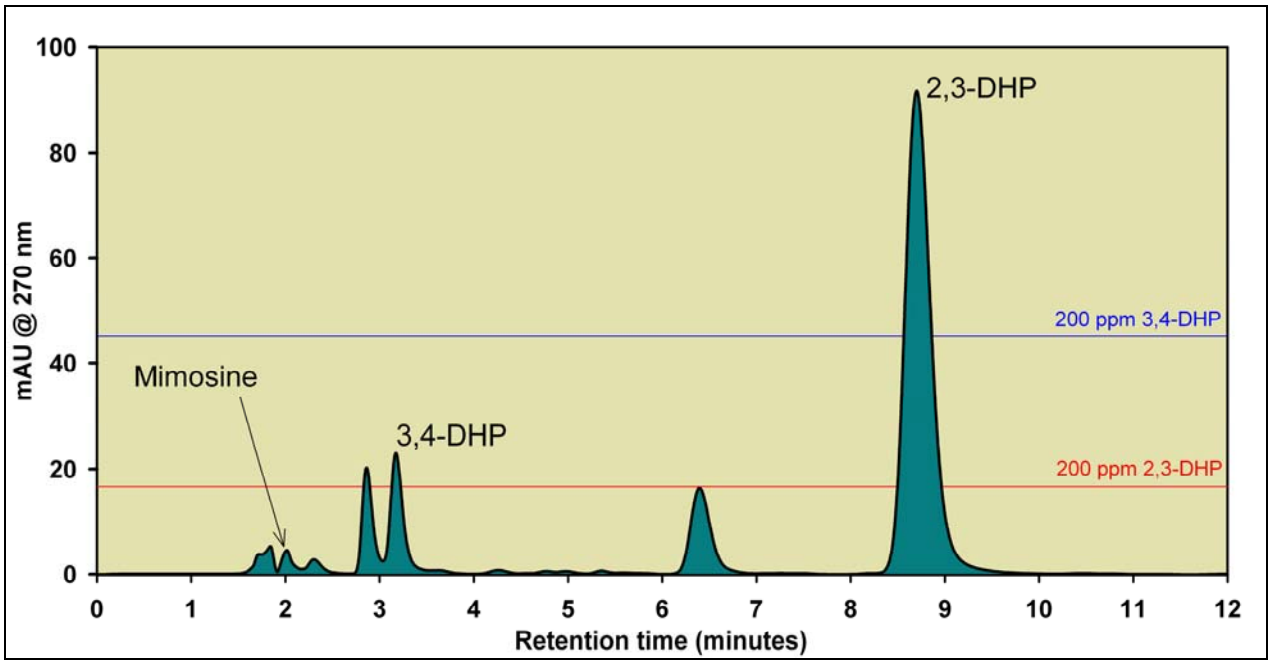

Figure 6: HPLC chromatogram of urine from a partially protected animal excreting high concentrations of 2,3-DHP.

These results contrast previous research findings that reported that 2,3-DHP was a transitory breakdown product (Ford *et al*. 1984) of 3,4-DHP degradation by rumen bacteria (Allison *et al*. 1992; Rincon *et al*. 1998 & 2000) and was often difficult to detect because it was assumed to be rapidly degraded soon after it was produced (Jones *et al*. 1985a). A number of strains of rumen bacteria, other than *S. jonesii*, have been identified that can degrade 2,3-DHP but not 3,4-DHP (Hammond *et al*. 1989; Allison *et al*. 1990; Galindo *et al*. 1995). The reason why ruminal populations of both these bacteria and *S. jonesii* were not efficiently degrading 2,3-DHP in cattle in the present study remains unclear. Detailed *in vitro* studies of the mechanisms by which *S. jonesii* degrades 2,3-DHP using cell-free extracts have revealed that the pyridine ring is enzymatically reduced by the bacteria, and that this enzymatic activity is induced by the presence of both 2,3-DHP and 3,4-DHP (Rincon *et al*. 1998). Degradation of 2,3-DHP occurred rapidly at the end of log-phase growth of *S. jonesii in vitro*, however degradation was inhibited by high concentrations of both DHP isomers indicating that a threshold concentration of enzyme was required to facilitate degradation (Rincon *et al*. 2000). Depriving *in vitro S. jonesii* cultures of a supply of 2,3-DHP for periods of 2 months or more has resulted in the temporary and irreversible loss of 2,3-DHP degrading activity (Dominguez-Bello *et al*. 1997; Rincon *et al*. 2000). Similarly Jones *et al*. (1985a) reported that one of four *in vitro* cultures of rumen fluid containing *S. jonesii* lost the capacity to degrade 2,3-DHP after just 6 days storage under anaerobic conditions. Therefore, the unexpected 2,3-DHP toxicity observed in the current study may be due to the temporary or permanent loss of 2,3-DHP degrading capacity from the rumen microflora of cattle in these herds. However, in contrast to the *in vitro* studies, good persistence of 2,3-DHP degrading activity by rumen bacterial populations have been observed *in vivo* in cattle deprived of leucaena for periods of time ranging from 6 months to 3 years (Jones *et al*. 1985b; Hammond

et al. 1989). An alternative hypothesis is that rumen microflora in these herds were in a dynamic growth stage and had not yet reached the critical population size or enzyme concentration required to trigger efficient 2,3-DHP degradation (R. Jones, personal communication). Follow-up testing of animals in these herds is required to validate this hypothesis.

Very few cases of elevated urinary concentrations of 2,3-DHP have been reported in cattle grazing leucaena at pasture (Jones *et al*. 1985a) and the threshold concentrations (urinary or serum) of 2,3-DHP required to induce toxicity in cattle are not known. Further detailed studies of 2,3-DHP toxicity in cattle and its effect on animal performance are required urgently.

4.2.2.2.5 Variation in urinary toxin concentrations within herds

There was substantial variation in the urinary concentrations of the toxins 3,4-DHP and 2,3-DHP between animals within the unprotected and partially protected herds (Appendix 4). The proportion of animals within each unprotected herd suffering 3,4-DHP toxicity was 100, 100, 90, 89, 80, 75, 40, 25 and 10%. The number of partially protected herds with >50%, 20-50% and <20% of animals excreting potentially toxic 2,3-DHP concentrations was 4, 6 and 4 respectively. This variation occurred despite the fact that the animals within each herd had consumed diets containing similar proportions of leucaena. Assuming the variation within herds was a real effect, and not an artefact of the experimental methodology employed (see 4.2.4), one possible explanation could be that each animal tested within the herds had a different population of microbes inhabiting its rumen. The interactions (competition, predation or synergism) between the bacteria responsible for DHP degradation and the other rumen microflora might explain why different animals were more or less efficiently protected from leucaena toxicity. Another possible cause for this variation could be ineffective passive animal-to-animal transfer of the rumen bacteria responsible for detoxifying the DHP isomers within each herd.

4.2.3 Relationships between environmental, animal and management factors and herd toxicity status

No clear links between location, climatic factors, class of animal or herd management practices (e.g. use of hormone growth promotants, feed supplementation, spells from leucaena etc) and the leucaena toxicity status of the herds were found in this study. Logistic regression analysis found that only the inoculation method used to introduce the bug into the herd was weakly, but significantly (P<0.05), related to herd protection status. A Chi squared and Fisher's exact test (P=0.596) revealed that more of the protected herds had been inoculated by direct drenching with the bug cultured by DPI&F than was expected if there was no association between these factors. Of interest, more of the partially protected herds than was expected had been inoculated by borrowing and mixing 'protected' animals thought to be carrying the bug. These data suggest that mixing 'protected' animals as a means of introducing the bug to the herd may predispose the herd to 2,3-DHP toxicity. A possible explanation for this observation could be that the capacity of *S. jonesii* and other bacteria to degrade 2,3-DHP can be lost in carrier animals (herds) overtime (as discussed above) and/or that 2,3-DHP degrading bacteria are not easily transferred from carrier animals to the rest of the herd. However, other studies have found 2,3-DHP degrading bacteria have persisted in cattle not grazing leucaena for 1-3 years and then rapidly and easily spread from carriers to recipient animals (Hammond *et al*. 1989; Allison *et al*. 1990), contradicting this hypothesis. More research into the rumen ecology of *S. jonesii* and other important 2,3-DHP degrading bacteria is needed to answer these questions. A key finding was that direct drenching with the DPI&F *S. jonesii* culture was common amongst protected herds, reinforcing that it is the most reliable method of introducing the bug to a herd.

4.2.4 Limitations to the interpretation of the results

4.2.4.1 Effects of dynamic bacterial population on toxicity status

This study provided a 'snapshot' or single observation of the toxicity status of the herds tested. There is evidence that the rate of excretion of 3,4-DHP and 2,3-DHP is dependent upon the size

and activity of the populations of the rumen bacteria responsible for the sequential degradation of these toxins (Ford *et al*. 1984; Rincon *et al*. 2000). Therefore, some of the cattle that tested positive to 2,3-DHP toxicity in this study may in fact have been protected and were only temporarily excreting high concentrations of 2,3-DHP while rumen bacterial populations adjusted to the levels of substrate being produced (R. Jones, personal communication). However, the design of the experiment to only test cattle that had been grazing leucaena for >3 weeks prior to sampling should have provided enough time for the rumen microflora to equilibrate prior to testing to minimize these effects. Indeed, many of the cattle in the unprotected herds suffering subclinical 3,4-DHP toxicity had been grazing leucaena for >3 months. Furthermore, the widespread occurrence of 2,3-DHP toxicity in herds from many different districts and management regimes suggest it is unlikely that all these cases are artefacts of fluctuations in rumen microbial populations.

4.2.4.2 Urine volume effects on toxin concentrations

Urine volume is determined by glomerular filtration rate in the kidneys, which is affected by the hydration state of the animal. Water intake, metabolic rate, heat stress and other environmental factors determine animal hydration state. The volume of each urination event can vary by 3-4 fold over any 24-hour period (D. Poppi, personal communication) and therefore significantly alter the concentration of metabolites present in the urine. A measurement of total 3,4-DHP and total 2,3-DHP excreted/kg liveweight/d for each animal, calculated from total daily urine output (volume) determined by creatinine analysis (Bolam 1998), would have yielded more robust results. We attempted to measure creatinine in the urine samples prepared for mimosine/DHP HPLC analysis. However, mimosine and both isomers of DHP absorbed UV light strongly at the optimal wavelength (λ = 205 nm) for creatinine measurement and mimosine eluted at the same time as creatinine (G. Kerven, unpublished data). This prevented the concurrent measurement of creatinine in the urine samples using the mimosine/DHP HPLC analysis. A modified HPLC procedure needed to be developed to measure creatinine in urine samples that contain mimosine and/or DHP, however this activity was beyond the time and budget constraints of the project. Therefore, the urinary concentrations of the toxins recorded in this study will be influenced by factors affecting the urine output of the animals tested, such as the level of hydration, time since last urination and fullness of the bladder (Guyton & Hall 2000). It could be expected that urine concentrations of the toxins would be diluted in well hydrated animals that were urinating frequently and elevated in dehydrated animals or those that had not urinated for some time. These factors may explain some of the variability in urine concentrations of the toxins observed between animals within herds, despite the fact that the dietary leucaena intake of animals within herds was quite consistent. Sampling cattle as soon as they were removed from pasture was employed to minimize the impact of urine volume on toxin concentrations. Urine volume will have little effect on the relative concentrations of 3,4-DHP and 2,3-DHP within each sample, therefore the finding of a significant number of partially protected herds is valid.

Other methods used to detect leucaena toxicity or protections status include: 1) measuring toxin concentrations in blood serum (Hegarty *et al.* 1979); 2) monitoring blood thyroxine levels (Jones & Winter 1982, Hammond et al. 1989); 3) testing the capacity of rumen fluid to degrade mimosine and DHP *in vitro* (Hammond *et al*. 1989; Allison *et al*. 1990); and 4) using molecular markers to test for the presence of *S. jonesii* in rumen fluid (Klieve *et al*. 2002). These methods were not used in the current study for the following reasons. Collecting blood and rumen samples from cattle in commercial yards, most of which were not equipped with a veterinary crush, was considered an unacceptable risk to the health and safety of the research team. Blood thyroxine levels are well correlated with toxicity and animal performance (Jones & Winter 1982), however they vary tremendously between individual animals and are only useful for monitoring changes in toxicity status of the same beast(s) over time by repetitive sampling. Collecting anaerobic rumen fluid 'on farm' is logistically difficult (R. Jones, personal communication), hazardous to the animal and is considered an invasive procedure by research animal ethic committees, and therefore was an undesirable method for the current experiment. Furthermore, the use of PCR molecular marker technology to test for the presence of *S. jonesii* in rumen fluid is laborious and very

expensive (A. Klieve, personal communication) and the mere presence of the bug's DNA may not be indicative of effective microbial detoxification of DHP. Therefore, despite its limitations, urine analysis was chosen as the best method to expediently determine the toxicity status of a large number of cattle at a single point in time.

4.2.4.3 Interactions between diet composition and urine toxin concentration

This study found there was no correlation between the proportion of leucaena in the diet of individual cattle and the concentration of the mimosine, 3,4-DHP and 2,3-DHP in their urine, either grouped as a whole or within herds. This reflects the variability in urinary toxin concentrations within herds, and indicated that differences in the efficiency of ruminal degradation of these toxins were the overriding factor determining the toxicity status of individual animals. Another factor that may have contributed to the poor relationship between diet composition and urine toxin concentration could be the lack of synchronicity of the excretion of urine and faeces by cattle. The time taken from forage ingestion to faecal excretion ranges from 12-36 hours in cattle, while urine is usually only retained in the bladder for 1-3 hours in well hydrated animals (D. Poppi, personal communication). Therefore the composition of urine reflects recent blood plasma composition, while faeces represents the diet consumed at a significantly earlier point in time. However, it is unlikely the animals' diet would have changed dramatically in the short term in the present study, particularly considering the prolonged acclimation period prior to sampling.

5 Success in Achieving Objectives

5.1 The postal/telephone survey

The postal/telephone survey successfully fulfilled the objective of reporting the current level of awareness of leucaena toxicity, the prevalence of toxicity observed by graziers & the inoculation & herd management techniques currently being practiced. In addition, useful information about the area planted to leucaena & the production systems utilizing these pastures was collected. This information emphasised the increasing importance of leucaena-pastures as a forage resource for finishing cattle in northern Australia.

5.2 The herd testing study

The herd testing study successfully determined the toxicity status of a sample of Queensland cattle herds grazing leucaena pastures. It revealed that subclinical 3,4-DHP toxicity is likely to be limiting animal performance in at least 20% of the herds tested, confirming the research team's hypothesis. The surprising result that 32% of herds were only partially protected & were excreting high concentrations of 2,3-DHP, highlighted significant gaps in our current scientific understanding of the detoxification pathway(s) of DHP & the role of specific rumen bacteria in this process. The effect of these high levels of 2,3 DHP is unknown, however 2,3 DHP is reported to be an appetite suppressant, & is therefore likely to be reducing animal production below potential.

5.3 Overall project success

The project has successfully achieved all of its objectives. The project has revealed that leucaena toxicity is still a problem in Queensland, partly due to complacency in herd management by graziers & the ineffective delivery of detailed information to graziers about leucaena toxicity & its prevention by research & extension agencies. However, the study has demonstrated our incomplete understanding of toxicity management in Queensland herds. Many herds were found to be unprotected despite having followed recommended procedures. The herd testing study also revealed limitations to our current understanding of detoxification pathways, the rumen ecology of the bacteria involved in these metabolic processes, the mechanisms of

transfer/spread of the bacteria within herds & the impact that different herd management practices will have on the efficacy of these bacteria.

6 Impact on Meat and Livestock Industry

6.1 Impact on Meat and Livestock Industry – now

The immediate impact of the project has been to raise the awareness of the problem of leucaena toxicity amongst approximately 350 known leucaena growers through the survey process. The distribution of a summary of the project findings & a 'Leucaena Toxicity Prevention Fact Sheet' to over 215 graziers & extension staff who participated in the study has provided detailed information to assist graziers implement effective rumen inoculation & herd management strategies. A conservative estimate of the value of lost production resulting from subclinical DHP toxicity in 20% of the national herd grazing leucaena is >$1.8M/yr (assuming: total pasture area = 100,000 ha; annualized stocking rate = 1.6 ha/hd; annual LWG = 275 kg/hd/yr; toxicity causes a 30% reduction in LWG; value of LWG = $1.8/kg). Since a further 32% of herds are suffering from high levels of 2,3, DHP, a known appetite suppressant, the potential economic impact of DHP toxicity is likely to be much higher. Preventing such production losses by the education of graziers through this project will have significant economic impact on both individual graziers & the beef industry.

The challenge remains to extend the findings of this project to the broader leucaena-growing community. The research team has started this process by presenting the information generated by the project to >100 graziers who have attended 'Leucaena for Profitability & Sustainability' (LPS) short courses (conducted by The Leucaena Network, UQ & DPI&F) in 2004 & another 200 graziers who attended recent leucaena field days at Inglewood & Mt Garnet. Another series of LPS short courses planned for late 2005 & the upcoming MLA-funded leucaena growers' manual "*A Graziers Guide to Leucaena Establishment & Management*" (NBP.224), will further disseminate information about leucaena toxicity to producers. DPI&F staff will continue distributing the 'Leucaena Toxicity Prevention Fact Sheet' generated by the project to all graziers who order batches of *S. jonesii* culture in future.

6.2 Impact on Meat and Livestock Industry – in five years time

This study has revealed that the number of graziers planting leucaena & the area of leucaena pastures is increasing dramatically in northern Australia. Recent seed sales indicate that >10,000 ha will be established in 2005 (weather permitting), an annual increment of 10% over existing leucaena plantings. At this rate of expansion, there will be an additional >50,000 ha of leucaena under grazing by 2010. At present we estimate that >114,000 hd/yr graze leucaena & as new plantings mature & reach full production, a greater number of cattle will be finished on leucaena in the near future. Thus any toxicological impediment to the production potential of cattle grazing this resource will have an increasing economic impact on the beef industry. Whilst education of new leucaena growers about leucaena toxicity will be critically important to ensure that sound management practices are implemented, further technical understanding of bug ecology & management is required to ensure that herds are fully protected into the future. Healthy cattle, reaching their production potential are the best advertisement for further expansion of leucaena pastures in northern Australia.

7 Conclusions and Recommendations

7.1 Conclusions

Leucaena toxicity is still an important production issue in Queensland. Many graziers think they have a good understanding of leucaena toxicity & its prevention, however this perceived knowledge is not being translated into the adoption of recommended rumen inoculation & herd management protocols. This study has found that subclinical DHP toxicity is likely to be limiting animal performance in 20% of the herds tested, & a further 32% of herds appear to have toxic levels of 2,3 DHP. This indicates that leucaena toxicity is having a significant detrimental economic impact. Of concern, 6 of the herds suffering DHP toxicity had been inoculated with the bug & the graziers managing these herds thought they were protected. Graziers need to be provided with accurate information about leucaena toxicity, & methods of prevention, to enable them to adjust their herd management strategies to safeguard the health & productivity of their cattle. The discovery of a large number of partially protected herds exhibiting 2,3-DHP toxicity was surprising & has highlighted shortfalls in our current understanding of how these toxins are broken down in the rumen.

7.2 Recommendations

The following recommendations & future research priorities arose from the project.

1. An on-going extension program is required to enable current & future leucaena growers to diligently monitor their herds for leucaena toxicity & implement recommended herd inoculation & management protocols to prevent it.
2. Many graziers are unsure of the toxicity status of their herds & due to environmental or market constraints cannot always follow recommended toxicity prevention protocols. The development of a simple on-farm leucaena toxicity diagnostic test kit would be a valuable tool to enable farmers to regularly check the toxicity status of their herds & enable rapid intervention to overcome occurrences of subclinical toxicity. A simple colorimetric urine test kit is currently being developed at The University of Queensland.
3. Animal management in leucaena grazing systems is constantly changing as graziers seek to optimize the efficiency with which they utilize this resource. For example:
    - Buying & selling of cattle in fattening enterprises complicates management procedures designed to retain the leucaena bug on-farm. More needs to be known about the rate of spread within a herd & the manner of spread within different animal social groups in order to ensure that the bug is retained when herd composition is constantly changing;
    - Leucaena toxicity may not be a serious issue when grazing leucaena for short periods of time (>50 days for backgrounding purposes) because DHP toxicity is cumulative, with symptoms & LWG suppression becoming progressively worse with time. However, when backgrounding for longer periods (50-100 days), LWG losses in unprotected animals may become economically important. The rate of passive animal-to-animal transfer of the bug in these situations will be an important factor determining the efficiency with which high turnover herds are protected from leucaena toxicity; &
    - Feeding grain rations changes the chemical (e.g. pH) & biological (composition of microflora) environment in the rumen of cattle. The effects of these changes on the populations & activity of *S. jonesii* & other bacteria involved in mimosine & DHP detoxification are not known.

    To enable researchers to adjust recommended toxicity prevention protocols to suit emerging leucaena production systems, improved knowledge of herd management in relation to bug spread is required, & more detailed *in vivo* microbiological studies of *S. jonesii* are required to answer the following questions:

- By what mechanism(s) are the bug & other bacteria spread from animal-to-animal?
- How long does it take for the bug to spread from the 10% of inoculated animals to protect the entire herd at a commercial scale?
- Does feeding supplements (e.g. grain, urea etc) affect the efficacy of mimosine & DHP degradation by rumen bacteria?

4. The discovery of widespread occurrences of 2,3-DHP toxicity has further highlighted limitations to our current understanding of DHP degradation processes *in vivo* in the rumen, & the identity & ecology of the specific bacteria involved. Research into 2,3-DHP toxicity in cattle is required:
   - to quantify threshold concentrations for 2,3-DHP toxicity & the effects of toxicity on production; &
   - To identify the bacteria are involved in 2,3-DHP degradation & to understand their ecology.

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9 Appendices

9.1 Appendix 1 - Postal/telephone survey questionnaire

University of Queensland
Leucaena Toxicity Survey 2004

This survey is voluntary and we appreciate your participation.

1. Do you grow leucaena on your property?
   - □ Yes (Please continue)
   - □ No (Thank you for participating. Please write your name on this form and return it to UQ)

2. Prior to receiving this survey were you aware that leucaena is potentially toxic to grazing cattle?
   - □ Yes, I understand leucaena toxicity and its prevention
   - □ Yes, I had heard this but know little about it
   - □ No, I did not know this

3. What cultivars of leucaena are planted on your property(s)?
   □ Peru □ Cunningham □ Tarramba □ Unknown

4. In what year was leucaena **first** planted on your property(s)?_____

5. In what year was leucaena **last** planted on your property(s)?_____

6. What area of land is planted to leucaena on your property(s) (acres)?_____

7. What is the total size of your property(s) (acres)?_____

8. On average, how many animals graze leucaena pastures on your property(s) in a year?_____

9. What class of animal do you graze on leucaena?
   □ Steers/bullocks □ Breeders □ Fat cows/heifers □ Other (Please specify)_____

10. For what main purpose do you graze leucaena? □ Growing/backgrounding □ Fattening/finishing
    □ Weaning □ Autumn grazing □ Drought reserve □ Other (Please specify)_____

11. What symptoms of leucaena toxicity (if any) have you observed in your herd?
    (Please exclude suspected cases of other diseases (e.g. 3 Day) that occurred while grazing leucaena)
    - □ Hair loss
    - □ Excessive salivation
    - □ Low breeder fertility
    - □ Unexplained cattle deaths
    - □ Lesions/sores/blisters on skin or in mouth
    - □ Lower than expected or poor performance
    - □ Sensitivity to sunlight
    - □ None (go to Q 14)

12. Have these symptoms occurred □ regularly □ infrequently or □ rarely? (Please detail occurrences)

    _____

13. What percentage of the herd exhibited symptoms of toxicity?_____

14. The following post-slaughter abnormalities may be associated with leucaena toxicity: lesions (ulcers) of mouth, throat or stomach; liver damage/sclerosis; and enlarged thyroid glands.
    Have you ever received reports from the abattoir of animals with these symptoms?
    □ Yes (Please specify (optional)) □ No □ Unsure □ No comment

PTO→

15. Have you inoculated with the leucaena "bug" (*Synergistes jonesii*) obtained from DPI?
    - □ Yes
    - □ No (Please proceed to Q19)

16. In which year/s did you inoculate with the bug? (Please list all years)_____

17. How many animals were inoculated_____(head) out of a mob of_____(head) grazing leucaena?

18. What "dose rate" was used (millilitres of inoculum per head or number of head/bottle)?_____

19. Have you used any of the following other methods to **obtain &/or retain** the bug in your herd?
    - □ Transfer of trough water from nearby property with bug
    - □ Transfer of manure from nearby property with bug
    - □ Transfer of rumen fluid from animals protected by bug
    - □ Borrowing animals from nearby property that have been inoculated with bug
    - □ Mixing new animals with existing animals thought to have the bug
    - □ Other (Please specify)_____
    - □ No (go to Q21)

20. Do you believe that your inoculation methods have been effective in preventing leucaena toxicity in your herd?
    □ Yes □ No □ Uncertain

21. What liveweight gain do your cattle typically achieve when grazing leucaena (kg/hd/d)?_____

22. What type of supplements have you fed while grazing leucaena? (Please specify type & quantity (kg/hd/d))

Hay	_____	Grain	_____
Molasses	_____	Urea	_____
Other	_____	Protein meal	_____

    □ None (go to Q 24)

23. At what time of year or seasonal conditions do you feed supplements?_____

24. Do your animals experience spells (breaks) of greater than 4 weeks from grazing leucaena?
    □ No □ Yes (Please specify how long)_____

Additional comments/extra information:_____

_____

_____

Please list the names & addresses of other graziers you think might like to participate in this survey:

_____

_____

_____

_____

Thank you for participating in this survey.

Please return the survey to UQ in the envelope provided.

9.2 Appendix 2 - Herd testing questionnaire

Herd Testing Questionnaire 2004

1. When was the last significant rainfall event?
   Month _____ Amount _____

2. What is the pasture composition of the leucaena paddock being grazed?
   Legume content _____
   Grass content _____

3. How long have the animals been on leucaena? _____

4. How many animals are in the herd? _____

5. Animals: Class _____
   Age _____

6. What type of supplements, if any, have been fed?
   Hay _____ Grain _____
   Molasses _____ Nitrogen _____
   Other _____

7. Do you use HGPs? _____

8. What is the estimated LWG of the herd? _____

9. How would you rate the herds performance
   Above expected _____ Expected _____
   Below expected _____

10. What is the inoculation history of the animals sampled? _____
    _____

11. Any symptoms of toxicity while on leucaena?
    Tongue/body ulcers _____ Hair loss _____
    Deaths _____ Salivation _____
    Other _____ None _____

12. What proportion of the herd has shown symptoms? _____

13. Any other interesting information? _____
    _____
    _____

Sample Number _____ Date Sampled _____
Property Owner _____ Property Name _____
Address _____
_____
_____

9.3 Appendix 3 - Summary of postal survey responses

Question	Answer	Response
Q2. Were you aware that leucaena was potentially toxic?	1. Yes, I understand toxicity and its prevention 2. Yes, but know little about it 3. No, I didn't know this	125 (73.1%) 42 (24.6%) 4 (2.3%) (n = 171)
Q3. What cultivars of leucaena are planted on your property?	1. Peru 2. Cunningham 3. Tarramba 4. Unknown	80 (46.8%) 109 (63.7%) 81 (47.4%) 7 (4.1%) (n = 170)
Q4. In what year was leucaena first planted on your property?	Before 1980 1980-1985 1985-1990 1990-1995 1995-2000 2000-2004	11 (6.5%) 12 (7.1%) 18 (10.6%) 23 (13.5%) 64 (37.6%) 42 (24.7%) (n = 170)
Q5. In what year was leucaena last planted on your property?	Before 1980 1980-1985 1985-1990 1990-1995 1995-2000 2000-2004	1 (0.6%) 2 (1.2%) 4 (2.4%) 10 (5.9%) 39 (22.9%) 114 (67.1%) (n = 170)
Q6. What area of your property has been planted to leucaena?	Total of all respondents	47,600 ha 117,600 acres (n = 170)
Q7. What is the total size of your property/enterprise?	Total of all respondents	816,300 ha 2,016,300 acres (n = 170)
Q8. On average, how many animals graze leucaena pastures on your property each year?	Total of all respondents	Approx. 55,300 hd (n = 164)
Q9. What class of animal do you graze on leucaena?	1. Steers/bullocks 2. Breeders 3. Fat cows/heifers 4. Other (predominantly bulls and weaners)	154 (90.5%) 30 (17.9%) 53 (31.5%) 32 (19.0%) (n = 168)
Q10. For what purpose do you graze leucaena?	1. Growing/backgrounding 2. Fattening/finishing 3. Weaning 4. Autumn grazing 5. Drought reserve 6. Other (breeding, conditioning bulls & research)	56 (33.1%) 137 (81.1%) 26 (15.4%) 11 (6.5%) 20 (11.8%) 12 (7.1%) (n = 169)
Q11. What symptoms of toxicity have you observed in your herd?	1. Hair loss 2. Excessive salivation 3. Low breeder fertility 4. Unexplained deaths (excl. suspected disease cases) 5. Lesions/sores/blisters on skin or in mouth 6. Lower than expected or poor performance 7. Sensitivity to sunlight 8. None	55 (32.9%) 8 (4.8%) 4 (2.4%) 15 (9.0%) 3 (1.8%) 23 (13.8%) 3 (1.8%) 93 (55.7%) (n = 167)
Q12. How often have toxicity symptoms occurred?	1. Regularly 2. Infrequently 3. Rarely	5 (6.8%) 20 (27.0%) 49 (66.2%) (n = 74)

Q13. What proportion of the herd exhibited toxicity symptoms?	Average proportion reported by 58 respondents	17.5% (n = 58)
Q14. Any post-slaughter abnormalities associated with leucaena toxicity reported?	1. Yes 2. No 3. Unsure 4. No comment	7 (4.2%) 149 (90.3%) 6 (3.6%) 4 (2.4%) (n = 165)
Q15. Have you inoculated with the leucaena bug from DPI&F?	1. Yes 2. No	102 (63.0%) 60 (37.0%) (n = 162)
Q16. In which years did you inoculate with the bug?	The number of respondents who had inoculated 1. Once 2. Twice 3. Three times 4. Four times or more	 71 (70.2%) 23 (22.8%) 5 (5.0%) 3 (3.0%) (n = 102)
Q17. What proportion of the mob grazing leucaena was inoculated?	Average proportion reported by 100 respondents	16.1%
	Number of respondents inoculating <10% of mob (below DPI&F recommended rate of inoculation)	24 (24%) (n = 100)
Q18. What dose rate was used (ml/head)?	Number of respondents drenching with <100ml/hd (less than the DPI&F recommended dose rate)	8 (11.3%) (n = 71)
Q19A. Have you used any of the following methods to obtain the bug?	1. Transfer of trough water 2. Transfer of manure 3. Transfer of rumen fluid from protected animals 4. Borrowing animals from protected herds 5. None (no effort made to obtain the bug at all)	31 (51.7%) 15 (25.0%) 5 (8.3%) 30 (50.0%) 17 (28.3%) (n = 60)
Q19B. Have you used the following method to retain the bug?	1. Mixing new animals with existing animals thought to have the bug 2. No on-going management of the bug	93 (57.4%) 69 (42.6%) (n = 162)
Q20. Do you believe your inoculation methods have been successful?	1. Yes 2. No 3. Uncertain	88 (68.2%) 4 (3.1%) 37 (28.7%) (n = 129)
Q21. What LWG do your cattle typically achieve when grazing leucaena?	Average LWG for 82 respondents <0.5 kg/hd/d 0.5 - 0.75 kg/hd/d 0.75 - 1.0 kg/hd/d 1.0 - 1.5 kg/hd/d >1.5 kg/hd/d	1.05 kg/hd/d 0 16 (19.5%) 14 (17.1%) 47 (57.3%) 5 (6.1%) (n = 82)
Q22. What type of supplements do you feed your cattle while grazing leucaena?	Hay Molasses Grain Urea Protein meal Other None	6 (3.6%) 21 (12.4%) 18 (10.7%) 24 (14.2%) 14 (8.3%) 27 (16.0%) 98 (58.0%) (n = 169)
Q23. At what time of year do you feed supplements?	Winter/dry season/drought Year round	52 (73.2%) 13 (18.3%) (n = 71)
Q24. Do your animals experience spells (breaks) of greater than 4 weeks from grazing leucaena?	No Yes	84 (52.2%) 77 (47.8%) (n = 161)

9.4 Appendix 4 - Raw herd testing data

Herd No.	Animal ID	Concentration in urine (PPM or μg/ml)			% Leucaena in diet
		Mimosine	3,4 DHP	2,3 DHP	
1	1	13	13	10	32
	2	13	10	24	46
	3	14	9	25	57
	4	11	8	-	47
	5	10	6	8	31
	6	12	9	-	49
	7	13	7	-	43
	8	12	3	-	35
	9	13	9	-	32
	10	12	5	-	31
2	1	12	12	-	31
	2	14	4	-	37
	3	14	7	-	36
	4	14	3	-	29
	5	14	12	-	33
	6	22	9	-	41
	7	22	0	-	26
	8	23	1	-	31
	9	23	5	-	24
	10	23	0	-	33
	11	27	4	-	33
3	1	15	-	-	32
	2	14	8	-	21
	3	13	7	-	23
	4	14	9	-	21
	5	14	8	-	19
	6	13	18	-	23
	7	14	6	-	18
	8	13	14	-	31
	9	13	4	-	25
	10	13	11	-	35
	11	14	31	-	33
	12	14	18	-	29
4	1	13	6	19	21
	2	13	16	228	17
	3	14	46	703	15
	4	13	8	31	16
	5	13	8	42	17
	6	13	9	122	18
	7	13	4	17	18
	8	13	8	23	17
	9	13	16	50	19
	10	12	11	152	19
5	1	12	9	26	25
	2	11	13	70	37
	3	13	10	35	30
	4	11	10	-	29
	5	12	17	-	N/A
	6	12	11	9	27
	7	9	4	-	19
	8	11	7	13	17
	9	23	0	-	21
	10	22	1	0	23
	11	21	5	-	17

- = compound not detected N/A = not available

Herd No.	Animal ID	Concentration in urine (PPM or μg/ml)			% Leucaena in diet
		Mimosine	3,4 DHP	2,3 DHP	
6	1	22	13	-	11
	2	21	14	31	14
	3	12	13	24	15
7	1	11	30	55	67
	2	12	7	11	51
	3	12	130	239	61
	4	12	80	72	53
	5	11	15	16	50
	6	11	12	29	52
	7	13	32	389	79
	8	11	29	36	62
	9	12	106	187	58
	10	11	51	95	60
	11	11	40	87	51
8	1	11	52	183	5
	2	10	16	56	9
	3	9	122	155	9
	4	9	168	679	8
	5	8	29	47	5
	6	9	21	70	7
	7	9	23	73	4
9	1	9	275	37	42
	2	9	205	22	47
	3	9	312	26	44
	5	9	96	24	43
	6	9	305	15	45
	7	9	608	35	47
	8	11	1001	138	56
	9	9	75	17	43
10	1	10	151	32	53
	2	9	100	29	59
	3	8	28	12	57
	4	9	53	15	41
	5	9	23	-	51
	6	9	88	18	52
	7	10	160	35	66
	8	10	133	25	44
	9	9	113	29	44
	10	9	223	43	57
11	1	13	1041	553	37
	2	11	594	438	31
	3	13	490	224	35
	4	9	128	34	37
	5	10	484	174	34
	6	14	616	79	47
	7	13	520	55	40
	8	14	612	694	39
	9	15	950	213	38

- = compound not detected N/A = not available

Herd No.	Animal ID	Concentration in urine (PPM or μg/ml)			% Leucaena in diet
		Mimosine	3,4 DHP	2,3 DHP	
12	1	13	4	-	48
	3	13	9	18	50
	4	13	13	22	N/A
	5	12	7	-	45
	7	13	7	-	N/A
	8	14	16	28	52
	9	12	-	-	55
	10	12	6	-	51
13	1	13	251	126	36
	2	14	585	136	42
	3	13	383	204	37
	5	13	713	195	37
	7	13	680	232	30
14	1	12	34	16	63
	2	12	3	-	53
	3	12	9	-	52
	4	12	22	63	64
	5	11	14	-	37
	6	12	4	-	53
	7	10	19	8	48
	8	10	12	-	37
	9	10	7	-	61
	10	10	12	-	41
15	1	10	26	239	62
	2	18	157	1809	64
	3	11	36	105	48
	4	13	19	139	60
	5	14	70	358	50
	6	11	14	542	41
	7	16	30	70	69
	8	13	69	1090	50
	9	12	74	692	56
	10	11	15	-	72
	11	12	12	25	68
16	1	9	13	249	32
	2	7	5	109	32
	3	7	9	149	36
	4	8	8	511	36
	5	6	1	-	45
	6	6	1	32	39
	7	7	8	104	27
	8	8	9	372	46

- = compound not detected N/A = not available

Herd No.	Animal ID	Concentration in urine (PPM or µg/ml)			% Leucaena in diet
		Mimosine	3,4 DHP	2,3 DHP	
17	1	12	6	1454	36
	2	11	4	26	36
	3	11	26	1167	24
	4	12	39	1153	24
	6	11	11	81	27
	7	13	16	1573	32
	8	11	10	62	40
	9	12	29	377	34
	10	10	11	351	37
	11	11	14	87	37
18	1	10	7	16	53
	2	10	4	-	59
	3	10	8	13	54
	4	11	9	11	67
	5	11	12	15	57
	6	11	19	20	64
	7	10	5	-	43
	8	10	5	-	54
	9	11	16	12	57
	10	10	11	17	41
19	1	12	15	216	40
	2	12	23	623	49
	3	10	7	183	48
	4	10	20	619	44
	5	-	-	29	39
	6	9	13	278	46
	7	10	26	995	49
20	1	7	8	23	30
	2	7	9	25	19
	3	8	17	158	28
	4	7	9	49	18
	5	7	24	129	33
	6	6	24	238	24
	7	6	6	5	N/A
	8	8	14	33	42
	9	8	92	825	24
	10	8	21	42	29
	11	7	6	20	26
	12	6	1	11	24
21	1	10	7	348	55
	2	10	5	59	56
	3	10	7	326	58
	4	12	32	1330	34
	5	11	12	487	32
	6	11	11	301	36
	7	10	4	48	45
	8	12	26	668	32
	9	10	6	189	40
	10	10	9	263	47

- = compound not detected N/A = not available

Herd No.	Animal ID	Concentration in urine (PPM or μg/ml)			% Leucaena in diet
		Mimosine	3,4 DHP	2,3 DHP	
22	1	11	4	65	51
	2	10	5	46	36
	3	9	3	-	33
	4	9	5	-	30
	5	9	3	20	41
	6	9	17	268	32
	7	9	5	-	35
	8	10	27	781	34
	9	10	9	49	23
	10	9	3	-	42
	11	10	19	212	33
	12	10	9	331	31
23	1	10	11	-	45
	2	10	13	9	41
	3	9	15	13	35
	4	9	8	11	46
	5	9	36	11	32
	6	7	11	8	27
	7	8	45	14	35
	8	8	10	7	42
	9	7	10	-	47
	10	8	52	14	39
24	1	10	8	163	26
	3	9	7	27	18
	4	10	18	227	23
	5	10	22	122	24
	6	10	8	26	23
	7	10	40	57	23
	8	9	3	32	21
	9	10	5	65	26
	10	9	3	14	33
	11	9	3	26	N/A
	12	9	3	-	24
25	1	11	7	43	44
	2	12	5	403	54
	3	13	4	39	39
	4	13	7	28	35
	5	13	7	127	44
	6	13	12	94	40
	7	12	2	10	30
	8	27	3	14	33
	9	16	-	12	28
	10	14	6	78	28

- = compound not detected N/A = not available

Herd No.	Animal ID	Concentration in urine (PPM or μg/ml)			% Leucaena in diet
		Mimosine	3,4 DHP	2,3 DHP	
26	1	7	4	-	48
	2	7	3	32	50
	3	6	1	19	45
	4	-	-	-	N/A
	5	7	6	30	41
	6	8	3	9	N/A
	7	7	7	10	49
27	1	8	12	8	35
	2	8	11	23	28
	3	8	16	177	43
	4	8	11	181	51
	5	7	26	451	22
	6	7	10	183	39
	7	7	-	8	34
	8	7	25	269	27
	9	8	18	60	33
	10	7	11	111	27
	11	8	29	428	32
	12	-	-	8	36
28	1	12	6	61	26
	2	13	8	222	22
	3	12	11	86	34
	4	15	47	1266	38
	5	16	41	604	20
	6	12	2	23	29
	7	14	11	261	22
	8	13	6	131	19
	9	12	5	18	9
	10	14	8	81	24
29	1	10	12	15	22
	3	9	11	37	48
	4	8	3	14	36
	5	8	8	36	20
	6	8	16	45	26
	7	9	12	79	17
	9	9	16	45	29
	10	8	3	7	25
30	1	11	92	30	22
	2	11	44	13	38
	3	10	18	-	41
	4	4	13	-	11

- = compound not detected N/A = not available

Herd No.	Animal ID	Concentration in urine (PPM or μg/ml)			% Leucaena in diet
		Mimosine	3,4 DHP	2,3 DHP	
31	1	7	12	-	17
	2	6	5	-	20
	3	6	7	-	21
	4	7	12	-	29
	5	7	12	-	19
	6	10	7	-	18
	7	10	10	-	22
	8	9	8	-	17
	9	10	8	-	19
	10	10	15	-	21
32	1	13	28	39	5
	2	15	227	47	15
	3	16	127	32	17
	4	16	221	162	15
	5	16	72	48	12
	6	15	77	111	11
	7	14	208	83	11
	8	16	220	41	28
	9	11	39	16	12
	10	13	115	37	13
33	1	8	7	15	48
	2	9	16	26	48
	3	7	5	8	45
	4	8	9	9	49
	5	8	12	27	46
	6	8	8	19	55
	7	8	13	36	39
	8	7	7	22	61
	9	8	7	17	49
	10	7	5	14	50
34	1	7	1	-	29
	2	6	2	-	39
	3	6	2	16	34
	4	6	2	-	27
	5	7	6	-	25
	6	7	4	-	34
	7	6	2	-	33
	8	3	7	12	31
	9	-	-	-	52
	10	9	6	14	38

- = compound not detected N/A = not available

Herd No.	Animal ID	Concentration in urine (PPM or μg/ml)			% Leucaena in diet
		Mimosine	3,4 DHP	2,3 DHP	
35	1	11	170	776	21
	2	11	287	1127	25
	3	11	228	527	31
	4	11	250	177	30
	5	12	278	680	32
	6	12	158	731	35
	7	11	269	261	36
	8	11	215	452	33
	9	12	392	667	26
	10	11	406	1357	32
36	1	15	206	126	48
	2	14	113	35	47
	3	13	664	248	44
	4	14	434	603	51
	5	15	1798	135	48
	6	12	351	75	44
	7	13	621	118	47
	8	14	507	514	46
	9	14	1999	492	45
	10	12	1192	533	57
37	1	11	10	51	41
	2	11	7	142	50
	3	10	6	25	41
	4	10	5	-	36
	6	6	4	-	N/A
	7	11	20	10	44
38	2	11	7	39	52
	3	12	3	18	44
	4	12	6	20	44
39	1	11	23	15	41
	4	11	139	27	40
	6	11	478	73	46
	7	11	67	55	41
	8	11	101	57	39
	9	10	127	21	30
	10	10	354	75	44
	12	10	42	25	40
40	1	12	516	80	29
	4	12	507	37	33
41	1	9	12	-	48
	2	9	19	14	50
	3	9	6	-	46
	4	9	8	11	49

- = compound not detected N/A = not available

Herd No.	Animal ID	Concentration in urine (PPM or μg/ml)			% Leucaena in diet
		Mimosine	3,4 DHP	2,3 DHP	
42	1	8	98	84	27
	2	7	44	22	27
	3	8	65	67	20
	4	7	15	14	27
	5	6	28	17	22
	6	6	18	15	23
	7	7	44	53	24
	8	6	7	-	22
	9	6	13	12	22
	10	7	6	-	22
43	1	5	26	15	25
	2	12	26	21	21
	3	-	4	-	19
	4	11	35	30	24
44	1	10	9	-	18
	2	10	16	17	14
	3	10	7	12	24
	4	10	18	-	18
	5	10	8	-	14
	6	10	10	11	17
	7	10	22	18	17
	8	10	57	55	14
	9	10	10	-	19
	10	10	15	16	14
	11	10	13	-	9

- = compound not detected N/A = not available