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Unravelling *Leucaena leucocephala* toxicity:

Ruminant studies in eastern Indonesia and Australia

Michael James Halliday

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Abstract

The forage tree legume leucaena [*Leucaena leucocephala* (Lam.) de Wit ssp. *glabrata* (Rose) Zarate] is a high-quality ruminant feed vitally important as a source of protein for livestock production in tropical regions. However, the presence of the acutely toxic non-protein amino acid mimosine in leaves and seeds, and its breakdown to chronically toxic hydroxypyridones (DHP), was believed to limit its productivity and adversely affect animal health. The Australian development of a fermenter-cultured oral inoculum containing the DHP-degrading ruminal bacterium *Synergistes jonesii* in the 1980s was thought to overcome this issue; however, anecdotal evidence of leucaena toxicity symptoms occurring in inoculated animals remained. In response, a program of research was initiated to investigate the efficacy of *S. jonesii* as well as other methods of protection against toxicity in ruminants. Broadly, this involved two areas of research, namely the effectiveness of: (a) microbiological degradation via *S. jonesii*; and (b) non-microbial metabolic detoxification.

Key objectives were to: (a) determine the efficacy of inoculation with *S. jonesii* both within Indonesia and Australia; (b) profile the toxicity status of ruminants in eastern Indonesia; (c) investigate the impact of mineral supplementation on 2,3-DHP toxicity; and (d) determine the extent of *in vivo* conjugation of DHP and its role in protecting ruminants against toxicity.

Initially, an Indonesian study involving the transfer of rumen fluid from “protected” ruminants to naïve Bali bulls was conducted. Sequential monitoring of DHP levels in urine indicated that there was no effect of inoculation. Secondly, a controlled animal house experiment was conducted in Australia to measure the impact of high leucaena diets on production in naïve steers and the effect of inoculation with the commercial *S. jonesii* inoculum. Key findings were: (1) inoculation had no effect on total DHP excretion; (2) indigenous *S. jonesii* strains were present in naïve animals prior to inoculation; (3) DHP did not suppress thyroid hormone production; and (4) high levels of the isomer 2,3-DHP were present without accompanying signs of toxicity.

The leucaena toxicity status of ruminants in eastern Indonesia was then assessed in a survey across the four islands of Lombok, Sumbawa, Sumba and West Timor. A number of different strains of *S. jonesii*, including the ATCC type strain (78.1), were detected, however *S. jonesii* was always at low population levels and always accompanied by high levels of undegraded 2,3-DHP in urine. Despite this apparent failure of *S. jonesii* to degrade DHP, there was no observed impact on animal productivity or health. Accordingly,

to investigate non-microbial detoxification mechanisms, a feeding trial involving goats in Indonesia was conducted to investigate the effect of Iron(II) sulphate mineral supplementation as a possible pathway for reducing the toxicity of 2,3-DHP by chemically binding to the toxin. Key findings were: (1) despite high levels of 2,3-DHP ingested by goats, no clinical signs of toxicity presented; (2) mineral supplementation did not result in an improvement in animal production; (3) evidence of conjugation of 2,3-DHP was observed; and (4) goats consuming diets of 100% leucaena were highly productive.

Finally, the role and mechanism of conjugation was investigated. This involved a longitudinal assessment of the toxicity status and productivity of Indonesian Bali bulls on 100% leucaena diets. High liveweight gains were recorded even though the bulls were persistently excreting high levels of 2,3-DHP. Using HPLC and HDMS it was found that up to 97% of DHP was excreted as 2,3-DHP in conjugated form. The conjugate was identified as glucuronic acid, forming a 2,3-DHP-*O*-glucuronide. It was concluded that conjugation played the key role in preventing DHP toxicity, as conjugation of DHP not only increases the speed of clearance of toxins from the body, but also actively detoxifies the compound. As the primary mode of toxicity of DHP is as a strong ligand (chelating with essential metals, resulting in deficiencies), the attachment of glucuronic acid inhibits this effect. Further, as metal binding occurs via the vicinal (*O*,*O*) moiety, the isomerisation of 3,4-DHP (from mimosine) to 2,3-DHP results in a weaker ligand and is therefore inherently less toxic. While the isomerisation pathway is not fully known, the detection of mono-hydroxypyridines (HP), previously unidentified, using HPLC and HDMS, could indicate possible intermediates.

The findings of this research greatly advance the knowledge of how ruminants adapt to DHP in diet, with the innate mechanism of conjugation identified as the primary mode of detoxification.

It is concluded that microbial detoxification is not the main pathway for toxicity control of ruminants consuming leucaena, and that inoculation with *S. jonesii* may no longer necessary. However, appropriate management of ruminants being introduced to leucaena diets is necessary to allow the animals to adapt and build metabolic tolerance.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed papers

Halliday, MJ, Padmanabha, J, McSweeney, CS, Kerven, G, Shelton, HM (2013) Leucaena toxicity: a new perspective on the most widely used forage tree legume. *Tropical Grasslands – Forrajes Tropicales* **1**, 1–11.

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Conference abstracts

Halliday, MJ, Giles, HE, Dalzell, SA, McSweeney, CS, Shelton, HM (2014a) The efficacy of *in vitro Synergistes jonesii* inoculum in preventing DHP toxicity in steers fed leucaena-grass diets. *Tropical Grasslands – Forrajes Tropicales* **2**, 68–70.

Halliday, MJ, Giles, HE, Shelton, HM (2014b) The incidence of high levels of urinary 2,3-DHP in ruminants consuming *Leucaena leucocephala* without clinical signs of toxicity. In 'Proceedings of the 30th Biennial Conference of the Australian Society of Animal Production'. (Eds S Hatcher, GL Krebs, BWB Holman) p. 180. (The Australian Society of Animal Production: Canberra)

Halliday, MJ, Panjaitan, T, Dahlanuddin, Padmanabha, J, McSweeney, CS, Depamede, S, Kana Hau, D, Kurniawan, Fauzan, M, Sutartha, Yuliana, BT, Pakereng, C, Ara, P, Liubana, D, Edison, RG, Shelton, HM (2014c) Prevalence of DHP toxicity and detection of *Synergistes jonesii* in ruminants consuming *Leucaena leucocephala* in eastern Indonesia. *Tropical Grasslands – Forrajes Tropicales* **2**, 71–73.

Halliday, MJ, Shelton, HM, Kerven, G, McSweeney, CS, Padmanabha, J (2016) Leucaena toxicity — a new management strategy. In 'Proceedings of the Northern Beef Research Update Conference'. p. 154. (The North Australian Beef Research Council: Rockhampton, Australia)

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Contributor	Statement of contribution
Halliday, MJ (Candidate)	Conception and design (50%) Analysis and interpretation (45%) Drafting and production (60%)
Padmanabha, J	Conception and design (5%) Analysis and interpretation (5%) Drafting and production (5%)
McSweeney, CS	Conception and design (5%) Analysis and interpretation (10%) Drafting and production (5%)
Kerven, G	Conception and design (5%) Analysis and interpretation (10%) Drafting and production (5%)
Shelton, HM	Conception and design (35%) Analysis and interpretation (30%) Drafting and production (25%)

Contributions by others to the thesis

This thesis is the original work of Michael Halliday. Additional contributions were made by Associate Professor Max Shelton, Dr Scott Dalzell, Dr Chris McSweeney, Dr Jennifer Waanders, Mr Graham Kerven, Ms Delma Greenway, Mr Peter Isherwood, Mr Jagadish Padmanabha and Ms Hayley Giles, as listed below.

- Halliday, MJ was responsible for 40% of the studies conception and design, 90% of the field work, 10% of laboratory analysis, 90% of data analysis and interpretation, 95% of drafting and writing and 90% of the final editing.
- Shelton, HM was responsible for 20% of the studies conception and design, 5% of data interpretation, 40% of the editing and 10% of the final editing.
- Dalzell, SA was responsible for 5% of the conception and design (of the Australian steer feeding trial) and 20% of the editing.
- McSweeney, C was responsible for 20% of the studies conception and design and 20% of the editing.
- Waanders, J was responsible for 10% of the laboratory analysis and 10% of the editing.
- Kerven, G was responsible for 10% of the studies conception and design and 10% of the editing.
- Greenway, D was responsible for 5% of the statistical analysis.
- Isherwood, P was responsible for 40% of the laboratory analysis.
- Padmanabha, J was responsible for 40% of the laboratory analysis, 5% of drafting and writing (of the microbiological sections).
- Giles, H was responsible for 10% of the field work, 5% of the conception and design (of the Australian steer feeding trial).

Statement of parts of the thesis submitted to quality for the award of another degree

Chapter 5, preliminary data on urinary DHP concentration and intake was used in a thesis submitted October, 2010 for partial fulfilment of the Bachelor of Agricultural Science (Animal Science) Degree Program, degree awarded 2010 within the School of Agriculture and Food Sciences. The thesis was awarded a grade of 6.

Research Involving Human or Animal Subjects

All research involving animals was reviewed by the UQ University Animal Ethics

Committee with ethical clearance obtained prior to commencement of experiments and monitoring. A copy of the ethics approval certificates are included as appendices 1–5. These include:

- **SAFS/144/11/ACIAR**: Investigations into DHP toxicity in livestock consuming leucaena in Queensland and eastern Indonesia and the potential presence of new DHP degrading bacterium or alternate strains of *Synergistes*.
- **LCAFS/035/10**: Investigations into degradation of mimosine by *Synergistes jonesii* in beef cattle herds grazing leucaena in Queensland.
- **AFS/160/12/ACIAR**: Investigations into the effect of 2,3- DHP toxicity, common in goats fed leucaena in Indonesia.
- **SAFS/023/15/ACIAR**: Investigations into DHP toxicity in livestock consuming leucaena in Queensland and eastern Indonesia.
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Keywords

synergistes jonesii, dihydroxypyridine, hydroxypyridone, mimosine, urine, rumen fluid, pcr, *leucaena leucocephala*, conjugation

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Dedications

In loving memory of my grandfather Jim Keary, whose inquisitive mind, passion for discovery and wonderful nature encouraged me to embark on this journey.

Table of Contents:

List of tables

List of figures

List of plates

List of abbreviations used in the thesis

=O	Keto/carbonyl moiety
–OH	Enol/hydroxyl moiety
2-HP	2-hydroxypyridine AKA 2-(1*H*)-pyridone
2-Po	2-(1*H*)-pyridone
2,3-DHP	2,3-dihydroxypyridine AKA 3-hydroxy-2(1*H*)-pyridone
2,4-DHP	2,4-dihydroxypyridine AKA 4-hydroxy-2(1*H*)-pyridone
2,5-DHP	2,5-dihydroxypyridine AKA 5-hydroxy-2(1*H*)-pyridone
2,5-HPo	5-hydroxy-2(1*H*)-pyridone
2,6-DHP	2,6-dihydroxypyridine AKA 6-hydroxy-2(1*H*)-pyridone
3-HP	3-hydroxypyridine
3,2-HPo	3-hydroxy-2(1*H*)-pyridone
3,4-DHP	3,4-dihydroxypyridine AKA 3-hydroxy-4(1*H*)-pyridone
3,4-HPo	3-hydroxy-4(1*H*)-pyridone
3,5-DHP	3,5-dihydroxypyridine
3,6-DHP	3,6-dihydroxypyridine AKA 3-hydroxy-6(1*H*)-pyridone
3,6-HPo	3-hydroxy-6(1*H*)-pyridone
4-HP	4-hydroxypyridine AKA 4-(1*H*)-pyridone
4-Po	4-(1*H*)-pyridone
4,2-HPo	4-hydroxy-2(1*H*)-pyridone
6,2-HPo	6-hydroxy-2(1*H*)-pyridone
ADF	Acid detergent fibre
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BD	Benton Dickinson
Bp	Base pairs
BW	body weight
CID	Collision induced disassociation
CP	Crude protein
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CTAB	Cetyltrimethylammonium bromide
d	day
D/HP	mimosine-derived pyridinyl metabolites (HP and/or DHP isomers)

DHP	Dihydroxypyridine AKA hydroxypyridone
DHP-*O*-G	DHP-*O*-glucuronide
DI	Deionised water
DM	Dry matter
DMD	Dry matter digestibility
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
ESI+	Electrospray injection positive
ESI−	Electrospray injection negative
Expt	Experiment
Fe	Iron
FTL	Forage tree legume
F.NIRS	Faecal near infrared reflectance spectroscopy
G.A.	Glucuronic acid
gDNA	Genomic deoxyribonucleic
GE	Gross energy
GLM	General linear model
Ha	Hectare
HB	Heating block
HCl	Hydrochloric acid
HDMS	High definition mass spectrometry
HP	Hydroxypyridine AKA pyridone
HP-*O*-G	HP-*O*-glucuronide
HPLC	High performance liquid chromatography
HPo	Hydroxypyridone
ICPOES	Inductively coupled plasma optical emission spectrometer
IFCC	International federation of clinical chemistry
LCMS	Liquid chromatography mass spectrometry
LECO	Laboratory Equipment Corporation
LSD	Least significant difference
LWG	Liveweight gain
MEGA	Molecular Evolutionary Genetics Analysis
Mg	Magnesium
MS	Mass Spectrometry

MS/MS	Tandem mass spectrometry
NDF	Neutral detergent fibre
NTB	West Nusa Tenggara
NTT	East Nusa Tenggara
O,O	Hydroxyl/carbonyl moieties
PCR	Polymerase chain reaction
PDA	Photo diode array
pFe	pFe = −log[Fe^{3+}]; the negative logarithm of the concentration of the free Fe(III) in solution, calculated for total [ligand]=10−5 M and total [iron] = 10−6 M at pH 7.4
pKa	acid dissociation constant
Po	Pyridone
QDAF	Queensland Department of Agriculture and Fisheries
qPCR	Quantitative polymerase chain reaction AKA real-time polymerase chain reaction
RCBD	Randomised complete block design
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
RP-SPE	Reverse phase solid phase extraction
RT	Retention time
s.d.	Standard deviation
s.e.	Standard error of the mean
SDS	Sodium dodecyl sulphate
SI	Selection index
SNP	Single nucleotide polymorphism
T_3	Triiodothyronine
T_4	Thyroxine
TFA	Trifluroacetic acid
TOF	Time of flight
UV	Ultra violet
WB	Water bath
wks	Weeks
Zn	Zinc

Chapter 1 General Introduction

1.1 Background to leucaena toxicity

Leucaena [*Leucaena leucocephala* (Lam.) de Wit ssp. *glabrata* (Rose) Zárate] is a multipurpose forage tree legume, widely used as a feed for ruminants in the tropical and sub-tropical world (Shelton and Brewbaker 1994). The many desirable traits of leucaena (including high crude protein content (Jones 1979); high palatability (Andrew *et al.* 2000); longevity; and tolerance to frequent severe defoliation and drought) have contributed to its success in ruminant production systems. Estimates indicate over 5 million ha have been planted worldwide (Brewbaker and Sorensson 1990), including more than 200,000 ha of leucaena-grass pastures in Australia (Dalzell *et al.* 2012).

Despite the many positive nutritional benefits of leucaena, it contains a toxic non-protein free amino acid, mimosine (β-[*N*-(3-hydroxy-4-oxopyridyl)]-α-aminopropionic acid) (Hegarty *et al.* 1976), which in high concentrations can severely affect animal health and performance. Ruminants are capable of converting mimosine to the less acutely toxic compound, hydroxypyridone (DHP), of which there are two common isomers [3-hydroxy-4(1*H*)-pyridone (3,4-DHP) and 3-hydroxy-2(1*H*)-pyridone (2,3-DHP)]. DHP is chronically toxic, reducing feed intake and animal performance (Jones and Hegarty 1984). Chronic DHP toxicosis is commonly referred to as 'leucaena toxicity'. Pioneering research into leucaena toxicity was conducted between 1976 and 1994 by R. J. Jones and colleagues (Hegarty *et al.* 1964a; Jones *et al.* 1976; Allison *et al.* 1992; Jones 1994). They published widely on the symptoms, chemistry, microbiology, and management of toxicity.

Mimosine, the toxin in leucaena, has anti-mitotic activity, affecting rapidly dividing cells (Tsai and Ling 1971; Jones and Hegarty 1984), causing symptoms including alopecia (Hegarty *et al.* 1964b), excessive salivation (Megarrity and Jones 1983), abortions (Holmes 1980), poor fertility (Holmes *et al.* 1981), and death (Jones *et al.* 1978). A compounding effect of mimosine is its strong affinity to bind metal ions, which can lead to severe essential element deficiencies (Tsai and Ling 1971). Toxicosis due to mimosine is relatively short term, and only appears when ruminants first consume leucaena or after substantial periods of time when leucaena is absent from the diet. This is due to the degradation of mimosine to DHP which occurs via plant hydrolase activity post-ingestion (Lowry *et al.* 1983) and many endogenous rumen microbes which are capable of rapidly and readily degrading the vast majority of mimosine consumed to DHP within 2 weeks from initial introduction (Hegarty *et al.* 1964b; Ghosh *et al.* 2007; O'Reagain *et al.* 2014).

The main toxin of concern in leucaena is therefore DHP, which is reported to cause goitrogenic effects in animals, reducing feed intake and liveweight gains (Hegarty *et al.* 1979; Jones 1979; Megarrity and Jones 1983), and it is also a potent ligand, causing essential element deficiencies (Tsai and Ling 1971; Ghosh and Samiran 2007). The initial metabolite of mimosine, 3,4-DHP, is commonly isomerised to 2,3-DHP, which is considered similarly toxic. In 1984, a gram-negative obligatory anaerobic bacterium was discovered in Hawaii, and later in Indonesia, subsequently named *Synergistes jonesii*, that was shown to be capable of degrading DHP to harmless by-products (Jones and Megarrity 1986). Degradation of DHP by *S. jonesii* was reported to involve the isomerization of 3,4-DHP to 2,3-DHP, followed by cleavage of the pyridine ring to unnamed pyruvates that are non-toxic and may have positive nutritional qualities (Allison *et al.* 1992). Rumen fluid from a Hawaiian goat containing *S. jonesii* was subsequently imported to Australia and used as the basis of a commercially available inoculum from 1984 (Quirk *et al.* 1988). Originally comprising rumen fluid, and later (1995 onwards) a fermenter-produced enrichment culture (Klieve *et al.* 2002), this inoculum was initially reported to protect animals grazing leucaena by degrading the majority of ingested DHP to harmless by-products.

However, recent findings worldwide have indicated the frequent occurrence of high levels of DHP in animals, which were otherwise considered protected by *S. jonesii*. Concerns regarding the efficacy of the inoculum were initially raised in 2004, following a survey of Queensland cattle which revealed high DHP levels in urine of animals grazing leucaena (Dalzell *et al.* 2012; Graham *et al.* 2013). Similar findings were subsequently recorded in ruminants in Thailand (Phaikaew *et al.* 2012), Mexico (Contreras-Hernández *et al.* 2013; Ruz-Ruiz *et al.* 2013), and Indonesia (Halliday *et al.* 2013; Halliday *et al.* 2014b) (Chapter 4), where animals were often fed high levels of leucaena in diet, up to 100%. Although shown to be excreting high levels of undegraded DHP, these animals did not present with any clinical signs of toxicity, nor was liveweight gain or reproductive performance affected. This anomalous yet widespread situation warranted further investigation of the efficacy of bacterial degradation with *S. jonesii*, and the possible role of alternate mechanisms for protection against toxicity, such as conjugation and chelation, which have not been the subject of in-depth research.

As leucaena is an important high-quality forage, the issue of uncontrolled DHP toxicity in ruminants represents a potentially serious impediment to animal productivity worldwide. Accordingly, this research program was initiated to investigate the efficacy of *S. jonesii* to degrade DHP, especially in high leucaena diets, and the role of alternate detoxification

mechanisms. The research focussed on the incidence of DHP toxicity and methods of detoxification by ruminants consuming leucaena in eastern Indonesia and Australia including: microbial degradation of DHP via the rumen bacterium *Synergistes jonesii* (Chapters 4, 5 & 6), and the role of an alternate metabolic pathway involving detoxification of DHP via conjugation in the liver (Chapters 7 & 8).

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Chapter 2 Review of literature:

Leucaena toxicity—a new perspective on the most widely used forage tree legume

The full paper can be downloaded from: https://doi.org/10.17138/tgft(1)1-11

2.1 Introduction

Leucaena is a multipurpose forage tree legume widely used in the tropical world (Shelton and Brewbaker 1994) with past estimates of up to 5 million ha planted worldwide (Brewbaker and Sorensson 1990). There are no current estimates but this area will have increased substantially (Dalzell *et al.* 2012). While first domesticated for human use 7000 years ago (Hughes 1998), its primary value is now as a feed for ruminants. Leucaena is high in crude protein (Jones 1979), highly palatable (Andrew *et al.* 2000), long-lived, and tolerant of frequent severe defoliation and drought. This latter feature is especially important, as it is able to provide edible forage long into the dry season (Shelton and Brewbaker 1994). Leucaena is a vitally important source of protein for ruminants throughout south-east Asia, China, India, Hawaii, the Pacific islands, Mexico, Central America, South America and Australia (Shelton and Brewbaker 1994). In tropical Australia, *L. leucocephala* ssp. *glabrata* cultivars were first released in the 1960s, and there are currently more than 200,000 ha of leucaena-grass pastures (Dalzell *et al.* 2012), with more plantings each year.

Factors such as the rapid increase in international demand for animal products, high cost of protein concentrates, and shortage of nitrogen for tropical grass pastures have increased the need for alternative high protein feed sources in the tropics. This has led to increased plantings of leucaena globally. The eastern Islands of Indonesia are just one of many examples, where leucaena is fulfilling an important role in ruminant production (Panjaitan 2012) and where its wider use is being promoted in Government programs.

However, while it has many positive nutritional benefits, leucaena possesses the toxic non-protein free amino acid mimosine in relatively high concentrations in leaves and young pods (up to 9% of dry matter (DM) in young leaves and 4–7% of DM in seeds) (Hegarty *et al.* 1964a). Pioneering work on leucaena toxicity was conducted between 1976 and 1994 by R. J. Jones and colleagues, who published widely on the symptoms, chemistry, microbiology and management of toxicity (Hegarty *et al.* 1964a; Jones *et al.* 1976; Allison *et al.* 1992; Jones 1994). They found that mimosine was rapidly converted to 3-hydroxy-4(1*H*)-pyridone (3,4-DHP) post-ingestion, which was reported to be a potent goitrogen

resulting in reduced feed intake, decreased liveweight gain (LWG), and poor animal performance on otherwise high-quality pasture (Jones and Hegarty 1984; Pattanaik *et al.* 2007). Jones and Hegarty (1984) reported that these symptoms occurred at intakes of leucaena >30% of diet.

Research conducted in the 1980s led to the isolation and identification of a previously unknown, and at the time unusual species of bacterium from the rumen of a Hawaiian goat; it was shown to be a gram-negative obligatory anaerobic bacterium and was subsequently named *Synergistes jonesii*. Strains from this species of bacterium were introduced into Australian cattle herds in 1983 and it was reported to provide protection against the toxic effects of leucaena by degrading DHP to harmless by-products (Jones and Megarrity 1986). This success was followed by the development of a commercial inoculum in Australia, comprised initially of rumen fluid (*in vivo*) and subsequently of fermenter-produced (*in vitro*) mixed inoculum containing *S. jonesii* (Klieve *et al.* 2002). Animals were reported to readily transfer the 'bug' within a herd (Quirk *et al.* 1988; Pratchett *et al.* 1991) (the exact method of transfer is unknown, but likely encapsulated in saliva or faeces), and it was recommended that only 10% of the herd need be inoculated. Protection of herds from toxicity in this way has led to annual LWGs of up to 300 kg in inoculated beef cattle grazing leucaena-grass pastures in northern Australia (Wildin 1994).

For these reasons, the problem of leucaena toxicity was considered resolved in Australia; however, a survey of the protection status of herds in Queensland on high leucaena diets in 2003 showed that almost 50% appeared to be unprotected, including previously inoculated herds (Dalzell *et al.* 2012). There was also poor understanding and awareness of leucaena toxicity by farmers, and ignorance of methods of detection of toxicity and its management to limit adverse effects. This was of great concern as undiagnosed subclinical toxicity was reported to lead to reduced animal production (Dalzell *et al.* 2012).

The aim of this review is to clarify the many issues regarding leucaena toxicity and to update readers regarding recent findings and future directions.

2.2 Occurrence of toxicity in tropical countries

During 1984–94, a survey of leucaena toxicity status, based on assay of urinary DHP excretions, was conducted in many countries where leucaena was being fed to ruminants, to determine the presence or absence of DHP-degrading bacteria (Jones 1994). R. J. Jones concluded that countries protected from toxicity by the presence of *S. jonesii* included: Indonesia, Vanuatu, Thailand, Malaysia, India, Seychelles, Mauritius and Mexico, while at least 13 other countries were not protected. He hypothesized that lack of protection in the latter countries may have caused an aversion to intensive feeding of leucaena and become a barrier to adoption, while countries protected from toxicity had a long history of use of leucaena as forage for ruminant production (Jones 1981). Thailand's inclusion as a protected country (Jones 1994) was based on the absence of urinary DHP in a single goat farm in 1983. However, a comprehensive survey of goat farms within 4 Thai provinces in 2009 (Phaikaew *et al.* 2012) found that all 63 goats sampled had exceedingly high levels of urinary DHP, often >1000 mg/L. Similarly, urine samples collected from goats consuming 100% leucaena in Mexico, also previously listed as a protected country, within the Yucatan region where leucaena is thought to have originated, had exceedingly high levels of DHP, in one case >10000 mg/L (Graham Kerven, unpublished data). Thus, it is not appropriate to refer to 'protected or unprotected' countries, as capability to degrade leucaena toxins varies among ruminants at the village and even farm level.

2.3 Confusion regarding the symptoms and molecular structure of the toxins

When leucaena was first introduced to Australia, the clinical symptoms of toxicity were thought to be quite serious, presenting as emaciated animals, and some animal mortalities in extreme cases (Jones *et al.* 1978). However, severe clinical symptoms are no longer common and most farmers feeding leucaena rarely report visible symptoms of toxicity. While some Australian graziers suspect subclinical toxicity by monitoring reductions in LWGs, livestock raisers in most other countries have no way of knowing if their animals are experiencing toxicity, as data are not available. Since their animals fed leucaena may still be performing better than those without legume, there is understandable denial that toxicity is an issue. Another highly anomalous situation is that many ruminants in the tropical world are consuming large amounts of leucaena for prolonged periods and excreting high concentrations of urinary DHP without any apparent clinical symptoms of toxicity (Phaikaew *et al.* 2012; Graham *et al.* 2013; Halliday *et al.* 2014c), including no indication of goitre (Palmer *et al.* 2010). The reasons for this are not understood, but may be due to alternative detoxification pathways such as chemical conjugation of the toxins, discussed later in this review.

2.3.1 Molecular structure of the toxins

The molecular structure of the toxins associated with leucaena are clarified. They are mimosine and its primary metabolites: 3-hydroxy-4(1*H*)-pyridone and 3-hydroxy-2(1*H*)-pyridone, commonly referred to as 3,4-DHP and 2,3-DHP, respectively (Figure 2.1). While mimosine and DHP are structurally similar, these compounds have different modes of toxicity and are largely responsible for different clinical and subclinical symptoms. It is therefore essential that toxicity symptoms are understood and the causes correctly identified for proper management of leucaena-based feeding systems.

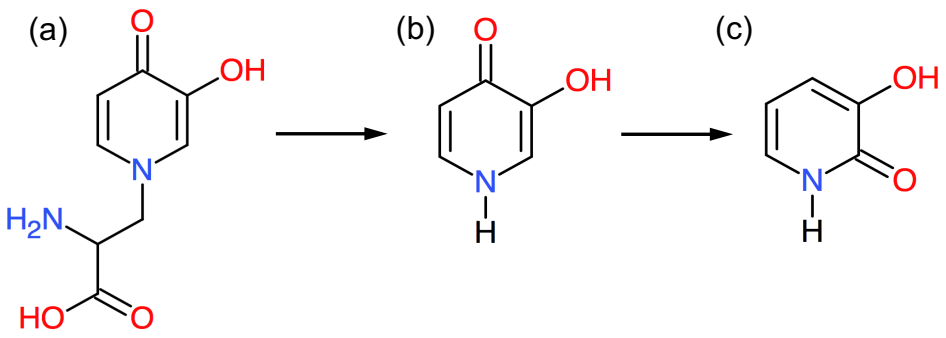

Figure 2.1: The ruminal degradation pathway of: (a) mimosine; (b) 3,4-DHP; and (c) 2,3-DHP, adapted from Hammond *et al.* (1989).

2.3.2 Mimosine toxicity

Mimosine (β-[*N*-(3-hydroxy-4-oxopyridyl)]-α-aminopropionic acid) (Hegarty *et al.* 1976) is acutely antimitotic, and inhibits the synthesis of DNA (Perry *et al.* 2005; Pandey and Dwivedi 2007), particularly in rapidly dividing cells (Tsai and Ling 1971; Jones and Hegarty 1984), and can cause damage to internal organs (Prasad and Paliwal 1989). The symptoms ascribed to mimosine include alopecia (Hegarty *et al.* 1964b; Ram *et al.* 1994), oesophageal lesions (Jones *et al.* 1978), foetal abortions (Holmes 1980), low bull fertility (Holmes *et al.* 1981) and death (Jones *et al.* 1978; Prasad and Paliwal 1989; Dalzell *et al.* 2012). It should be noted that mimosine itself is not responsible for the symptoms of goitre (Hegarty *et al.* 1979).

Structurally, mimosine (Figure 2.1a) is a tyrosine analogue (Hegarty *et al.* 1964b; Hashiguchi and Takahashi 1977), capable of inhibiting enzyme functions such as tyrosine decarboxylase and tyrosinase (Crounse *et al.* 1962). The inhibition of these enzymes, especially of [$^{3+}$]thymidine in the follicle bulbs of hair cells, along with the incorporation of mimosine into biologically vital proteins (Tsai and Ling 1971), can result in depilation of actively growing hairs.

For this reason, alopecia is one of the most commonly reported symptoms when animals are first introduced to leucaena, and can occur within 7 days of consuming 100% leucaena diets (Hegarty *et al.* 1964b). Hair loss is commonly observed from areas including the penis sheath and tail where growth is more continuous as mimosine affects the follicle bulb only in the active phase of growth (Hegarty *et al.* 1964b). This is best demonstrated in sheep as wool growth is largely continuous and therefore sensitive to alopecia from mimosine toxicity (Hegarty *et al.* 1964b). Alopecia is evident when levels of mimosine in diets are >0.015% body weight (BW) (Szyszka and Termeulen 1985). As a diet of 100% fresh leucaena leaves may result in consumption of as high as 0.031% BW mimosine (Tangendjaja *et al.* 1985), it would seem that leucaena feeding at levels greater than 50% would lead to alopecia in unprotected animals.

While mimosine toxicity can be potentially severe, it is relatively short-term and appears only when animals are first introduced to high leucaena diets (>50% leucaena) (Ghosh *et al.* 2007; O'Reagain *et al.* 2014). Ruminants typically acquire the ability to fully degrade mimosine within 2 weeks from initial introduction (Ghosh *et al.* 2007) and excretion of mimosine ceases within 24 hours of removal of leucaena from the diet (O'Reagain *et al.* 2014). For this reason, symptoms of mimosine toxicity rarely persist within an animal, especially if introduced to leucaena slowly (Jones 1979).

The degradation of mimosine to 3,4-DHP occurs via many endogenous rumen bacteria (Hegarty *et al.* 1964b) and by plant hydrolase activity of endogenous enzymes within leucaena leaves (Smith and Fowden 1966; Lowry *et al.* 1983). Up to 30% of the mimosine is converted to 3,4-DHP in the initial process of mastication (Ram *et al.* 1994) within 1 hour of ingestion (Jones and Megarrity 1983). Although mimosine is readily degraded to 3,4-DHP, this does not result in detoxification.

Mimosine also forms strong complexes with metal ions (Hashiguchi and Takahashi 1977). While the chelation of mimosine antagonizes its antimitotic ability (Hashiguchi and Takahashi 1977), the process of chelation with high affinity metal ions from animal cells, such as Fe(II), Cu(II) and Zn(II), inhibits many enzymatic pathways; these metals are also required for normal hair growth (Hegarty *et al.* 1964b; Hashiguchi and Takahashi 1977; Paul 2000). Ultimately, the chelatory effects of mimosine exacerbate the overall depilatory effects, resulting in overlap of some symptoms with DHP toxicity (Puchała *et al.* 1995).

2.3.3 DHP toxicity

The primary metabolite of mimosine is the compound 3-hydroxy-4(1*H*)-pyridine (3,4-DHP) (Hegarty *et al.* 1976), which, in the presence of certain ruminal microbes, can be further converted to its isomer 3-hydroxy-2(1*H*)-pyridone (2,3-DHP) (D'Mello 1992). Both isomers of DHP are reported to act as potent goitrogens, due to their antiperoxidase activity. By inhibiting essential peroxidase- and lactoperoxidase-catalyzed reactions (Christie *et al.* 1979; Lee *et al.* 1980), the iodination of tyrosine in the binding step of the thyroid is inhibited. This step is crucial for the synthesis of thyroid hormones, such as thyroxin (T_4) and triiodothyronine (T_3), resulting in depressed serum T_4 levels, which causes overstimulation and enlargement (up to 4 times) of the thyroid glands (goitre) (Hegarty *et al.* 1979; Jones 1979; Megarrity and Jones 1983). Studies have also shown that persistent administrations of DHP increase the uptake of iodine in the hyperplastic thyroid (Hegarty *et al.* 1979). One effect of lowered serum T_4 and T_3 levels is reduced appetite, and ultimately a decreased LWG. Serum T_4 levels below 13 nmol/L may even result in death (Jones *et al.* 1978).

Reduced iodine availability due to DHP can also affect the salivary glands. Iodine is incorporated into salivary glands via a different method and DHP inhibits the trapping step rather than the binding step, as in the thyroid (Koutras *et al.* 1967; Harden *et al.* 1968). This can lead to excessive salivation after prolonged exposure to leucaena (Jones *et al.* 1976; Jones *et al.* 1978; Holmes *et al.* 1981; Megarrity and Jones 1983; Ram *et al.* 1994).

Compounding the goitrogenic effects of DHP, is the fact that both isomers also strongly chelate with metal ions (Tsai and Ling 1971), forming complexes with Zn, Cu, and Fe in particular, leading to excretion and depletion of these elements (Ghosh and Samiran 2007). A deficiency in Zn was shown to be responsible for skin lesions (Mills 1978; Paul 2000), increased salivation (Mills 1978; Puchała *et al.* 1996) and abnormal hair growth (Hashiguchi and Takahashi 1977). Zn deficiency can also be responsible for inhibiting DNA replication (Perry *et al.* 2005) and can adversely affect spermatogenesis (Yamaguchi *et al.* 2009). These all suggest that the chelation of essential minerals is a major toxic effect of DHP. The presence of existing deficiencies of essential minerals in the diet may hasten the manifestation of clinical toxicity symptoms.

The severity of DHP toxicity is a function of both amount of leucaena in diet and time on leucaena. Clinical symptoms may take up to 8 weeks to become evident (Quirk *et al.* 1988). This can manifest itself in scenarios where new animals on a leucaena-grass paddock can gain up to 1 kg/head.day, while existing animals grazing that same paddock for longer periods can be gaining as little as 0–0.2 kg/head.day (Jones and Bray 1983). These lowered LWGs were found to be directly related to lowered levels of serum T_4, with a threshold of 25 nmol/L before clinical symptoms such as drooling and hair loss were observed (Jones and Winter 1982). It is this chronic nature of DHP, the dual modes of toxicity, and the long periods when toxicity can go unnoticed that contribute to the complexity of recognition of potential toxicity.

However, as mentioned, inexplicably, goats in Thailand and Indonesia on long-term high leucaena diets were shown to be excreting high concentrations of 2,3-DHP in urine without indication of goitre (Phaikaew *et al.* 2012; Halliday *et al.* 2014c). These exceptions will be discussed later in this review.

2.3.4 The dominance of 2,3-DHP

The isomer 2,3-DHP was originally thought to be transitory, and indicative of incipient ruminal colonization of *S. jonesii* (Ford *et al.* 1984; Jones *et al.* 2009). Much recent data from Australia, Thailand and Indonesia contradict the notion of 2,3-DHP being transitory (Dalzell *et al.* 2012; Phaikaew *et al.* 2012; Graham *et al.* 2013; Halliday *et al.* 2014c) and indicate that it is usually the dominant isomer in ruminants fed leucaena long-term. Phaikaew *et al.* (2012) reported exceedingly high levels (>1000 mg/L) of 2,3-DHP in the urine of ruminants fed over extended periods (>3 months). It is generally considered that the isomers 3,4-DHP and 2,3-DHP are equally harmful (Lee *et al.* 1980; Ghosh *et al.*

2008). The latter has been shown to reduce intake (suppress appetite) (McSweeney *et al.* 1984), reduce milk production in dairy cows (Ghosh *et al.* 2007), and be fatal given intraruminally in pure form (Puchała *et al.* 1995).

In a controlled leucaena feeding trial in India (Ghosh *et al.* 2007), 2,3-DHP was the dominant isomer excreted 21-days after commencement of feeding in cattle naïve to *S. jonesii*, as was the case for animals offered 25–100% leucaena in a controlled trial in Queensland (Halliday *et al.* 2014a).

The accumulation of high levels and proportions of 2,3-DHP in ruminants both previously exposed to *S. jonesii* and those naïve to it may suggest that: (a) rumen microbes other than *S. jonesii* are capable of degrading 3,4-DHP to 2,3-DHP; (b) the *in vitro S. jonesii* inoculum released in Australia may have lost/mutated the strains effective at complete DHP degradation; (c) there are other environmental factors (including regulation of genes involved in DHP metabolism) affecting optimal ability of *S. jonesii* to completely degrade all DHP; and lastly, (d) there may be alternative non-bacterial pathways for detoxification of DHP.

2.4 Taxonomy and distribution of *S. jonesii*

Recent advances in molecular techniques for the detection and sequencing of *S. jonesii* have allowed greater insights into the geographic spread and genetic variability of the bacterium. From the culture originally imported into Australia from a Hawaiian goat in 1982, 4 strains were later isolated, including the designated type strain 78.1 (ATCC 49833) and 3 others (100-6, 113-4, 147-1) (Allison *et al.* 1992). The strains were shown to have differential specificity for degrading mimosine, and/or 3,4-DHP and 2,3-DHP (Jones 1994).

PCR amplification using *S. jonesii* specific primers of the 16S rDNA (small sub-unit of the ribosome-ssRNA) was used as a molecular phylogenetic classifier, and has even identified *S. jonesii* 16S rDNA sequences in the gut of disparate animals such as the horse, Tamar wallaby, baboon and the emu (Chris McSweeney, unpublished data). The theoretical limit of detection of *S. jonesii* by PCR is 10^3 cells/mL; however, a realistic PCR amplification of genomic DNA from digesta is 10^4–10^5 cells/mL due to the co-precipitation of contaminating inhibitors. This problem was overcome by a second round of PCR (nested PCR) on the initial PCR products, which increased the sensitivity of detection to near theoretical limits. Sequencing of the amplified (nested) products not only confirmed the identity of *S. jonesii* but also detected mutations in that segment of the 16S gene. These changes appeared as discrete mutations or ‘single nucleotide polymorphisms’ (SNPs) that may be correlated with their ability to degrade DHP, relative to the type strain. SNPs can be random or occur consistently at the same locus, termed ‘hot-spots’.

A survey of animals consuming diets of 0–100% leucaena was conducted to understand the distribution and changes at the molecular level of *S. jonesii* (Chris McSweeney, unpublished data). Rumen fluid or faeces was collected from cattle in Queensland and from cattle, sheep, goats, buffalo and yak from Indonesia, Thailand, Vietnam, China and Brazil. In a number of these sites, animals were naïve to leucaena. In general, faecal samples failed to generate PCR products for *S. jonesii* 16S rDNA from either Australian or international samples. However, the survey revealed that *S. jonesii* is far more widespread than originally realized. The molecular detection of this bacterium internationally in ruminants, irrespective of their leucaena feeding status strongly suggest it is an indigenous rumen microbe.

This information on its ubiquity has come with the proviso that the sequence analysis from Australia and other countries showed SNPs across ~800 of the 1500 bases of the 16S gene of the bacterium. These SNPs were distributed primarily at ‘hot-spots’ in bases

corresponding to *E. coli* nucleotide positions 268 (C→T), 306 (A→G), 328 (G→A) and 870 (A→C) between bases 200–900 (~700 base pairs (bp)) of the *S. jonesii* ATCC 16S rDNA. All 4 SNPs were identified at varying frequencies in all farms surveyed in Queensland. Of these loci, '306' and '870' were almost always mutated when SNPs were detected. Surprisingly, these two SNPs were present consistently in the inoculum provided to the farmers. In about 50% of these sequences, all 4 SNPs were present.

In animals overseas, the very same SNPs were also distributed, ranging from frequencies of 15% (for '870' in Brazilian cattle) to 100% (all 4 SNPs in Vietnamese cattle and goats). Among all the international samples analysed, those from Jinnan cattle, Tibetan yak and Indonesian buffalo were found to have *S. jonesii* that was 100% identical to the 78.1 type strain. Interestingly, the buffalo in Indonesia were on 100% leucaena for 0.5–1 years and had high clearance of both 3,4-DHP and 2,3-DHP. The Jinnan cattle and Tibetan yak were naïve to leucaena. Other SNPs were detected, whose frequencies were not consistent across animals, geographical regions or loci.

It remains to be determined if these molecular changes also modify the ability of these strains to degrade DHP. A detailed molecular and physiologic analysis of these 'strains' is highly desirable in order to elucidate the link between genetic changes and DHP-degrading potential.

Attempts at molecular enumeration of *S. jonesii* from rumen digesta are similarly fraught with ambiguity, relating to the sensitivity and specificity of the quantitative real-time PCR (qPCR) technique. Compared with the 2-step nested PCR which produces ~800 bp long products, the qPCR typically uses a 100–200 bp PCR product for real-time amplification and analysis. Although this method has a statistically higher chance of amplifying a shorter target (100–200 bp) than the nested PCR (700–800 bp), it still has to overcome PCR inhibition and thus a lack or reduction of sensitivity, unlike the nested PCR. Any specific amplification, nevertheless, can accurately enumerate the target molecule (16S rDNA of *S. jonesii*) from a rumen sample. Several primer sets targeting the *S. jonesii* 16S DNA, designed and tested during the past 4–5 years, have indicated *S. jonesii* exists in the rumen at very low numbers, even in animals foraging on leucaena and where degradation of DHP was apparently occurring (Graham *et al.* 2013).

This extensive molecular analysis of animals consuming leucaena demonstrates that *S. jonesii* is widespread with variants of the ATCC type strain, but in low numbers, regardless of quantity of leucaena in diet. Nevertheless, these variants may potentially be responsible for the partial DHP degradation results.

2.5 Management of toxicity

2.5.1 Methods for detecting DHP toxicity

A full understanding of DHP toxicity and of effective methods to detect subclinical toxicity is vital for productive management of animals on leucaena. There are several possible approaches to detection of toxicity, but the most effective method is assay of urine for DHP (Lowry *et al.* 1985; Phaikaew *et al.* 2012). Unmetabolised mimosine, 3,4-DHP and 2,3-DHP are readily absorbed into the blood stream and voided in the urine via glomerular filtration at the kidneys (Hammond 1995). While small amounts (up to 15%) may be voided in the faeces (Hegarty *et al.* 1979; Jones and Hegarty 1984; Hammond 1995), most is readily absorbed from the gut and excreted via the kidneys (Lowry *et al.* 1985).

While HPLC analysis of urine (Dalzell *et al.* 2012) is currently the most accurate method of measuring DHP toxicity, sampling many animals for analysis by HPLC is too expensive for graziers, and prohibitive for farmers in developing countries. In response to the need for a rapid, accurate and inexpensive urine assay, a colorimetric test kit, modified from earlier work (Lowry *et al.* 1985), has been developed and refined as the most cost-effective and immediate assessment of toxicity (Graham *et al.* 2013). This is now available to Australian graziers uncertain about the toxicity status of their herds. This improved urine test gives reliable, although not quantitatively precise, indications of the concentration and nature of the toxin.

DHP can be excreted in both the free form and as a conjugate. HPLC analysis with a reduced flow rate has been able to separate free DHP from the conjugated DHP-glucuronide. However, preservation of urine prior to analysis requires strong acidification, which hydrolyses all conjugated DHP (Tangendjaja and Wills 1980), unless analysis is conducted immediately.

While assay of serum T_4 and T_3 can be used as an indicator of DHP toxicity (Jones *et al.* 1978; Megarrity and Jones 1983; Jones and Hegarty 1984; Ghosh *et al.* 2007), changes in thyroxin levels occur after longer-term exposure with considerable variation among animals (Michael Halliday, unpublished data). Likewise, the development of goitre in response to depressed thyroid hormones is also a cumulative longer-term effect. As noted, ruminants do not always develop enlarged thyroids on high leucaena diets. Accordingly, these methods are not a reliable indicator of current toxicity status.

2.5.2 Inoculation

Following the initial discovery of *S. jonesii*, inoculation with rumen fluid collected from protected animals was the preferred method for transferring protection between herds, and even between countries (Jones *et al.* 1985b). This seminal work on inoculation demonstrated that the capability to completely degrade DHP was transferred within 5 days following direct inoculation with rumen fluid (Jones and Lowry 1984; Jones and Megarrity 1986). In 1982, 10 cultures of rumen fluid from a single goat in Hawaii were imported to Australia and, in 1983, dosed into rumen-fistulated steers at the CSIRO Lansdown Research Station near Townsville (Jones and Megarrity 1986). Rumen fluid from these steers was then used as the source of inoculum and given via direct rumen injection, to ~10% of the remaining herd at Lansdown. It was then thought to have spread passively to the entire herd. In 1984, strained rumen fluid from these cattle was administered to steers at the Queensland Government Brian Pastures Research Station near Gayndah (Quirk *et al.* 1988). It was from these 2 locations that rumen fluid was collected and distributed to Australian graziers and also to livestock raisers in both Ethiopia and China, with reported successful transfer of protection (Jones 1994).

In 1995, production of the inoculum was begun in an *in vitro* fermenter, using rumen fluid containing the mixed bacterial inoculum sourced from cattle at Brian Pastures (Klieve *et al.* 2002). While originally successful in transferring protection, the efficacy of the current *in vitro* inoculum appears in doubt, as it is neither rapid nor completely successful in its degradation of DHP (Graham *et al.* 2013). At the time, it was thus postulated that the continually cultured oral inoculum may have lost some strains, and/or have undergone genetic modification and/or contained other strains with an altered DHP-degrading potential (Graham *et al.* 2013; Halliday *et al.* 2014a).

2.5.3 Other microbial control options

Other microbial solutions apart from *S. jonesii* have been investigated as alternative biological control methods. Domínguez-Bello and Stewart (1991) reported the isolation of a DHP-degrading *Clostridium* bacterium. In China, 4 strains were isolated, which together were able to degrade up to 60% of DHP *in vitro* within 3 days. These gram-positive facultative and obligate anaerobes were identified as *Lactobacillus* spp., *Streptococcus bovis* and *Clostridium sporogenes* (Tan *et al.* 1994); and were quite different from *S. jonesii*. Strains of obligate aerobic *Streptococci* capable of degrading DHP were reported by Chhabra *et al.* (1998). Researchers in Germany isolated a mimosine- (and DHP-)

degrading bacterium from the rumen fluid of steers naïve to leucaena (Aung *et al.* 2011). After continuous culture with mimosine for 16 days, their work reported the isolation of an aero-tolerant gram-negative coccobacillus belonging to the genus *Klebsiella*. While capable of growing under anaerobic conditions, it grew best in aerobic conditions. As such, it may not readily persist in high numbers in the anaerobic conditions of the rumen. The researchers also described a method for stabilising the inoculum in alginate beads, increasing its shelf-life at room temperature by up to 8 weeks. Methods such as this, and freeze-drying, may be incorporated into the *in vitro* production of *S. jonesii* inoculum in the future, thus preventing loss of bacterial numbers in transit. However, further research is required.

2.5.4 Supplementation and conjugation

As mentioned, one of the major toxic effects of DHP is to strongly chelate essential minerals such as Fe and Zn (Stunzi *et al.* 1980). Methods for increasing conjugation and/or chelation of DHP by modifying the diet have been postulated as a way to reduce the toxic effects of leucaena.

While Christie *et al.* (1979) reported DHP conjugated with glucuronic acid to have reduced antiperoxidase activity in vitro, Hegarty *et al.* (1979) showed that the conjugate was of the same order of chronic goitrogenicity *in vivo*. However, chelation and conjugation increase the polarity of DHP, resulting in a more water-soluble molecule, which can be voided more efficiently. In addition, the attachment of a glucuronic acid molecule or metal ion at an active binding hydroxyl site, reduces the toxic chelating ability (Lowry *et al.* 1985).

While DHP can be excreted in the free form, it is normal for up to 33% to be found conjugated as a glucuronide in urine (Hegarty *et al.* 1964b). Supplementation with molasses has previously been shown to increase both the conjugation of DHP, and the amount being excreted in unprotected animals (Elliott *et al.* 1985). Animals receiving a high molasses supplement did not exhibit a decline in T_4 levels. However, the level of molasses required to achieve this effect was ~ 40% dry matter intake, making it an impractically large component of the diet.

Supplementation with minerals to chelate mimosine and DHP has been suggested as a method of detoxification (Hashiguchi and Takahashi 1977). Supplemental metal ions were shown to have prevented hair loss and skin lesions (Jones *et al.* 1978), doubled excretion of intraruminally infused 2,3-DHP, lowered the levels of 2,3-DHP in the rumen and plasma, and prevented clinical toxicity symptoms from developing (Puchała *et al.* 1995), suggesting

that kidney clearance of DHP is more efficient when chelated or conjugated (Puchała *et al.* 1995). While DHP is more efficiently voided in the chelated form, if essential metal ions are not replaced, deficiencies in these elements will result in toxicity symptoms (Puchała *et al.* 1996).

2.5.5 Feeding management

Management strategies that control exposure to leucaena have been effective in limiting the extent of toxicity. Prior to the discovery of *S. jonesii*, Jones and Hegarty (1984) suggested that leucaena should not exceed 30% of the diet. The current common management practice of Australian graziers is still to feed leucaena at ~30% of diet, which is also considered an optimal as a protein supplement. As mimosine toxicity rarely occurs in animals regularly consuming leucaena and since DHP toxicity is a factor of both time on leucaena and amount of leucaena in the diet (Hammond 1995), moderate levels of leucaena in the diet for short periods (≤2–3 months) can limit the toxic effects. Quirk *et al.* (1988) showed that it can take up to 8 weeks for clinical symptoms to become evident. Alternating time on leucaena with time on other feed sources might be a way to reduce the toxic effects associated with DHP. However, reducing consumption of leucaena may also limit the LWG potential of a fattening system. Given that leucaena is often the only high protein feed available during the extended dry season in many tropical smallholder feeding systems, an effective biological control mechanism appears to be the most practical solution to leucaena toxicity. Other less effective management strategies include drying leucaena. While this converts most of the mimosine to DHP (Hegarty *et al.* 1964a; Wee and Wang 1987), it does not overcome the toxic effects of DHP itself.

The best practice management guidelines for feeding of leucaena in Australia (Dalzell *et al.* 2006) are current as of 2006 and exclude the new findings of this thesis. The recommended intake of leucaena is between 35–40% leucaena in diet, which practically involves managing the time animals spend in a leucaena paddock by rotational grazing, and also the planting density of the leucaena rows, to provide an overall balance of approximately 30–40% leucaena in the paddock. Graziers are currently advised to inoculate 10% of their herd with the commercial inoculum, allowing for reported full coverage of the herd within 5–6 weeks and to maintain carrier animals on leucaena to enable the transfer of *S. jonesii* after destocking. It is important to note that these best practices are targeted towards steers for meat production, where any potential negative effects on reproduction are not considered critical. There is less knowledge on the management of breeders, especially if naïve to leucaena, and graziers are advised to

avoid introducing pregnant cows to leucaena within the first trimester, due the possible effects of mimosine as they adapt to leucaena in diet.

It is the aim of this thesis to provide updated recommendations for the best management of animals consuming leucaena in Australia.

2.6 Conclusions

Despite the many nutritive benefits of leucaena, the antinutritional properties of mimosine and its DHP-metabolites have the potential to affect animal health and production and limit wider adoption of leucaena in ruminant-based feeding enterprises. The discovery of a bacterium capable of degrading DHP, *S. jonesii*, led to greater adoption in Australia where it has been distributed as an inoculum, however concerns regarding toxicity persist, both in Australia and internationally where an inoculum is not available.

Although leucaena toxicity has been researched for over 30 years, many knowledge gaps still remain. These include the method of transmission of inoculated *S. jonesii* within a herd; the now apparent indigenous nature of *S. jonesii*; the reason for high levels of urinary 2,3-DHP, without apparent negative affect; and the importance of non-microbial detoxification mechanisms. Additional clarification is also required regarding the main method of toxicity of DHP, given that goitre is now rarely observed, and to the relative toxicity of both DHP isomers.

Conflicting evidence with the original research include the finding that, while high concentrations of DHP were reported to be harmful, many ruminants worldwide consuming high levels of leucaena are productive and healthy despite incomplete degradation of DHP, with 2,3-DHP the common isomer. This, coupled with the findings that *S. jonesii*: (a) may not be fully effective on high leucaena diets; (b) appears not to persist in the rumen at high numbers; and (c) has much genetic variation between geographical regions, suggests alternate protection mechanisms are therefore important in overall animal protection against DHP toxicity. These may include molecular transformation of the DHP compound to reduce its toxicity without degradation, such as hepatic conjugation or metal-ion chelation.

As transfer mechanisms involved in bacterial control of toxicity are beyond the resources of many countries, and the persistence and efficacy of the Australian inoculum is of concern, the international significance of an alternate control mechanism cannot be understated. As such, improved understanding and management of leucaena feeding systems to minimise any negative effect due to DHP is vital to promote the increased uptake of this extremely productive legume.

Chapter 3 Scope of thesis

3.1 Thesis objectives

The overall objective of this thesis was to investigate methods for overcoming leucaena toxicity in ruminant feeding systems in eastern Indonesia and Australia, with the purpose of improving feeding efficiency and animal productivity; and ultimately farmer livelihoods. The thesis comprises two broad topics of research: microbial degradation and metabolic detoxification. The former topic followed the long-held understanding that toxicity was overcome by the action of *S. jonesii*; the latter topic investigated an alternative pathway that toxicity was overcome by hepatic conjugation.

The specific objectives for microbial degradation included:

- Investigation of the feasibility and efficacy of inoculation of Indonesian ruminants using fresh rumen fluid from 'protected' animals (Chapter 4).
- Investigation of the efficacy of the fermenter-cultured *S. jonesii* inoculum in Australia, for control of leucaena toxicity (Chapter 5).
- Survey of DHP toxicity status and *S. jonesii* prevalence in ruminants in leucaena-based feeding systems in eastern Indonesia (Chapter 6).

The specific objectives for metabolic detoxification include:

- Investigation of the impact of 2,3-DHP toxicity on the productivity of weaner goats, and the feasibility of detoxification by chelation with mineral supplementation (Chapter 7).
- Optimisation of the method of hydrolysis of conjugated DHP for HPLC analysis and identification and quantification of the conjugate using HDMS and HPLC (Chapter 8).
- Determination of the extent of conjugation of DHP as a detoxification mechanism in new and existing steers fed 100% leucaena diets in eastern Indonesia (Chapter 8).

3.2 Thesis hypotheses

The overall hypothesis of this thesis evolved during the period of study, based on the significance and contradictory nature of emerging results when compared to the previous understanding of toxicity as outlined in the earlier literature. Accordingly, a new hypothesis was postulated that redefines the understanding of leucaena toxicity worldwide. The general hypothesis at commencement was: In Indonesia, distribution of *S. jonesii* would be sporadic, isolated to areas where leucaena was being fed and that animals in these areas would be protected by functional *S. jonesii*; and in Australia the commercially produced cultured inoculum was effective in controlling leucaena toxicity.

The final two chapters were conducted with a redefined general hypothesis: *S. jonesii* is not capable of affording complete protection to ruminants on high leucaena diets, and animals are protected against DHP toxicity by the process of hepatic conjugation of DHP.

The original specific hypotheses were:

- Transfer of fresh rumen fluid from 'protected' donor animal to recipient animals in eastern Indonesia would quickly confer DHP degrading capacity (Chapter 4).
- The commercial fermenter-cultured inoculum containing *S. jonesii* would be effective in degrading all DHP in Australian steers on high leucaena diets (Chapter 5).
- In remote regions and islands in eastern Indonesia, where animal movement is restricted, the spread of the bacteria may be impeded, and animals may not be protected by *S. jonesii* (Chapter 6).

The specific hypotheses for the final two chapters were:

- The primary mode of DHP toxicity is the strong affinity of the hydroxyl groups to bind with metal ions, and that supplementation with minerals would reduce the toxic effects of DHP (Chapter 7)
- The low populations of *S. jonesii* are insufficient to fully degrade all DHP in animals consuming high levels of leucaena and they adapt to high leucaena diets by the process of conjugating DHP in the liver (Chapter 8).
- The conjugate compound is the glucose-based carboxylic acid: glucuronic acid (Chapter 8).

Chapter 4 Effectiveness of inoculation with rumen fluid containing *Synergistes jonesii* to control DHP toxicity in ruminants in eastern Indonesia

4.1 Introduction

Leucaena leucocephala (leucaena) is a productive forage tree legume which contains the toxic non-protein amino acid mimosine. While this secondary plant compound can seriously affect animal health, in ruminants, rumen microbes rapidly convert mimosine to hydroxypyridone (DHP) which is chronically, rather than acutely toxic. This occurs post-ingestion via a combination of rumen microbes and endogenous plant enzymes (Hegarty *et al.* 1964b; Lowry *et al.* 1983). The widely-published method of protection against DHP toxicity is via microbial degradation by a specialized rumen bacterium, *Synergistes jonesii*, strains of which, including the type strain (78-1), can degrade 3,4-DHP to 2,3-DHP and further to non-toxic by-products (Allison *et al.* 1992). Evidence now suggests *S. jonesii* is to be considered a microbe indigenous to the rumen, with discrete mutations or variants detected in DNA gene sequences associated with different geographical locations. Variants of *S. jonesii* have been identified in ruminants in Indonesia (Padmanabha *et al.* 2014). These variants may account for varying capacity of *S. jonesii* to degrade DHP (Padmanabha *et al.* 2014).

Seminal work on leucaena toxicity research has demonstrated that functional *S. jonesii* can be rapidly and readily transferred from 'protected' animals to susceptible animals using rumen fluid inoculum (Jones and Lowry 1984). In eastern Indonesia, there are vast distances separating 'protected' animals from those susceptible to toxicity, including between islands. For microbial protection against leucaena toxicity to occur, a practical method is required to successfully transfer rumen fluid from 'protected' to 'unprotected' ruminants, which may be in remote locations and often of a different species (e.g. goats, buffalo, *Bos indicus* and *Bos javanicus* cattle).

Accordingly, this trial was conducted to investigate the feasibility and efficacy of inoculation using rumen fluid from 'protected' buffalo on Sumba island that were consuming high leucaena diets but were excreting low levels of urinary DHP, when transferred to cattle and goats in nearby West Timor island. These latter animals were excreting high levels of urinary DHP and were thus not fully degrading DHP. As an inoculum does not currently exist in Indonesia, apart from the effectiveness of inoculation, it was important to evaluate the feasibility of collecting, storing and transporting rumen fluid anaerobically, with subsequent inoculation of smallholder animals as a possible long-term solution for controlling DHP toxicity in ruminants fed leucaena in eastern Indonesia.

4.2 Materials and Methods

The experiment was conducted on the islands of Sumba and West Timor in eastern Indonesia, over an 18-day period beginning in December 2012.

Donor animals were two female buffalo (*Bubalus bubalis*) from Melolo, Sumba. These buffalo were selected from a herd where *S. jonesii* had been detected (Chapter 6) (Halliday *et al.* 2014c; Padmanabha *et al.* 2014) and were excreting low urinary DHP (<20 mg/L DHP) while consuming >70% leucaena diets. Recipient animals were selected from areas known to be prone to DHP toxicity (Chapter 6), including: three kacang goats (*Capra hircus*) from Sumlili, Timor; and four Bali bulls (*Bos javanicus*) from Bone, Timor. All were consuming high leucaena diets (50–100% leucaena), and were excreting urinary DHP at levels >1800 mg/L DHP. Three cattle (*Bos javanicus*, two bulls and one cow) from Tarus, Timor, previously consuming low leucaena diets (<30% leucaena), were also inoculated. All animals were maintained on diets of 100% fresh cut leucaena throughout the monitoring period. Recipient animals were randomly allocated to receive either the Sumba rumen fluid inoculum or a control treatment consisting of a water drench (Table 4.1).

Table 4.1: Number of animals allocated to each treatment.

Treatment	Melolo, Sumba buffalo (*n*)	Sumlili, Timor goats (*n*)	Bone, Timor cattle (*n*)	Tarus, Timor Cattle (*n*)
Sumba donors	2			
Sumba inoculum		2	1	1
Control		1	3	2

Urinary DHP concentration was estimated following colour development using the iron(III) chloride method (Plate 4.1) (Graham *et al.* 2014). Buffalo urine was spot sampled daily for 3 days prior to collection of rumen fluid, while donor animals were monitored 2–4 times during the 8 days pre-inoculation, with urine collected 5 and 10 days post-inoculation. Methodology for hydrolysis of conjugated DHP to the free form was adjusted by omitting boiling for 1 hour (Graham *et al.* 2014), and instead allowing samples to hydrolyse over several days at ambient temperature (up to 37ºC). During this period the colour intensified, indicating hydrolysis of conjugated DHP consistent with Graham *et al.* (2014). Approximate concentrations of free DHP were estimated based on colour hue and intensity (Plate 4.1); the overwhelming majority of samples were dominated by the isomer 2,3-DHP.

Rumen fluid for inoculation was collected via an orogastric tube (Graham *et al.* 2013), strained through muslin cloth, pooled, mixed and stored in a glass thermos and glass Schott® bottles. Inoculation occurred on the same day as collection, including travel between islands, and was administered via an orogastric tube. All containers were initially flushed with rumen fluid, and young fresh ground leucaena leaves were added as a mimosine substrate. The bottles were kept at ~37ºC and an anaerobic environment was maintained via the active fermentation of leucaena. Doses were: goats from Sumlili—100 mL/head; bulls from Bone—240 mL/head; and cattle from Tarus—150 mL/head. The trial was sanctioned under animal ethics approval # SAFS/144/11/ACIAR (Appendix 1).

Repeated observations with time were analysed using Minitab® statistical software 16 (©2010, Minitab Inc., State College, PA, USA) with a General Linear Model ANOVA using a split-plot design (with treatment as the main-plot and time as the sub-plot; individual animals as a random factor, nested with treatment and location).

Colour development	Approximate 2,3-DHP concentration (mg/L)
	0
	400
	1000
	2000
	3000

Plate 4.1: Examples of colour hues from the colorimetric test with estimated 2,3-DHP concentration.

4.3 Results and Discussion

There was no effect due to animal species or location on urinary DHP levels (P = 0.84); thus, treatment means were assessed as an average of all animals receiving each treatment. There was no interaction effect between treatment and location (P = 0.70). Mean urinary DHP levels in the two donor buffalo from Sumba, from 5 samples collected, were low (<20 mg/L DHP). Although the scale of this study did not afford molecular analysis of rumen fluid samples, the buffalo belonged to the same herd which previously tested positive for *S. jonesii*, and as the animals were on up to 100% leucaena diets, it was assumed that functional *S. jonesii* was present in the rumen of these animals. In contrast, since mean urinary DHP levels in recipient animals, tested up to four times each pre-inoculation, were high (Table 4.2), exhibiting normal variation (Giles *et al.* 2013; Graham *et al.* 2013), it was concluded that these animals were not 'protected' by rumen bacteria. The overwhelming majority of DHP was excreted as the isomer 2,3-DHP. Despite having originally been thought to indicate the commencement of degradation by *S. jonesii*, the presence of 2,3-DHP is now common, and doesn't necessarily indicate effective degradation is occurring.

There were no significant differences in mean urinary DHP levels between animals dosed with the Sumba inoculum and the control treatment, either pre- or post-inoculation (P = 0.50) (data not presented). Variation within treatment groups was high, most likely due to the differential adaptation of individual animals to high levels of leucaena within their first month on the diet. Despite the lack of a treatment effect, ten days post-inoculation, urinary DHP levels in all animals, while still high, had declined. This however did not reach statistical significance compared with pre-inoculation levels (P = 0.12), likely due to the small sample size. DHP levels remained above the considered safe threshold of 100 mg/L DHP (Dalzell *et al.* 2012). This result contrasted with earlier reports where transfer of protection following inoculation with rumen fluid was rapid and complete with urinary DHP levels declining within 5 days in Indonesia (Jones and Lowry 1984) and within 3 days in Thailand (Palmer *et al.* 2010). However, while Jones and Lowry (1984) reported a "dramatic" decline in DHP excretion after inoculation, the 2 dosed goats in the experiment were already degrading 45–62% of ingested DHP prior to inoculation. This natural decline in DHP excretion and apparent inherent capacity to partially degrade ingested DHP was common in both the past and the present study.

Since it is known that *S. jonesii* exist in the rumen in low populations, often below the limits of detection by nested PCR (<10^4–10^5 cells/mL) (Graham *et al.* 2013), it was possible that

the dosed inoculum did not increase *S. jonesii* populations sufficiently to achieve protection. Although *in vitro*, the study of McSweeney *et al.* (1993b) required 2 weeks for *S. jonesii* to establish a competitive population capable of 3,4-DHP degradation, quantified as abundance of *S. jonesii* RNA in a mixed-culture chemostat. Therefore, this study may have terminated too soon. Doses in this study were limited by the volume of rumen fluid collected from the buffalo, and although dosage volumes were less than used in the work of Jones and Lowry (1984) (350 mL), they were greater than used by Palmer *et al.* (2010) (30 mL). As *S. jonesii* was neither confirmed nor quantified in this study, it was possible that the selected donor animals did not have effective *S. jonesii* populations. Possible alternative explanations for the low urinary DHP include the diurnal variation of urine volume and toxin metabolism and the possibility of a period of low leucaena in diet.

Table 4.2: Mean (±s.e.) urinary DHP excretions and 95% confidence intervals from all animals as estimated by iron(III) chloride test. Due to a lack of significant treatment effect, treatment means are not separated.

Average of all animals	Mean DHP (mg/L)	95% CI	*n* (samples)	*n* (animals)
Pre-inoculation	1676 ± 276	(948, 2402)	22	10
5 days post-inoculation	1580 ± 444	(852, 2308)	10	10
10 days post-inoculation	824 ± 322	(96, 1552)	10	10

4.4 Conclusions

In summary, rumen fluid collected from buffalo in Sumba assumed to contain functional *S. jonesii* and given to unprotected ruminants in Timor did not rapidly and completely transfer protection within 10-days post-inoculation. Increasing time on 100% leucaena diets appeared to lead to a natural decline in urinary DHP excretion; however, this failed to reach significance ($P > 0.10$). While limited by the length of monitoring, the results of this study suggest a different outcome following inoculation procedures from that was originally reported over 30 years ago.

This study has also highlighted the technical and logistical difficulties involved in collecting and transferring *S. jonesii* anaerobically as a method of inoculation against DHP toxicity, especially in multi-island countries where supply-chain systems are limited. For this reason, it is concluded that the methodology evaluated in this study was not practically suitable for eastern Indonesia. The equipment and skills required to complete these actions are not currently available in the Indonesian Government livestock and extension services, and would require specialist training, the uptake of which would be problematical. Further work to develop suitable techniques is warranted, including exploring the possibility of transferring animals to allow the natural spread of rumen bacteria in a herd.

The decline in urinary DHP levels in control animals indicated an inherent ability to adapt to DHP. Possible adaptation mechanisms include the presence of a less-functional variant of *S. jonesii*, or metabolic detoxification of DHP by conjugation. Although the method of acid hydrolysis of DHP has been improved to optimise colour development in iron(III) chloride reagent (Graham *et al.* 2014), it is possible that the decline in DHP levels could be attributed to incomplete hydrolysis. Any remaining conjugated DHP would be unable to bind with Fe in the solution, thus preventing the development of colour representative of the total DHP in urine.

Further work should aim to study: (a) the presence and functional capacity of *S. jonesii* throughout the multi-island provinces in eastern Indonesia; and (b) the possible role of conjugation in detoxification. At a practical level, finding an alternative control mechanism to eliminate the need for inoculation would greatly benefit these systems.

Chapter 5 The efficacy of a cultured *Synergistes jonesii* inoculum to control hydroxypyridone toxicity in *Bos indicus* steers fed leucaena/grass diets

The full paper can be downloaded from: https://doi.org/10.1071/AN17853

5.1 Introduction

The pioneering work on *Leucaena leucocephala* (leucaena) toxicity, conducted in the 1980s, reported the unique capability of the gram-negative ruminal bacterium *Synergistes jonesii* to rapidly and almost completely degrade the hydroxypyridone (DHP) toxins in ruminants consuming high leucaena diets (Jones and Megarrity 1983; Allison *et al.* 1992). The first step in the degradation process was reported to be the isomerization of 3,4-DHP to 2,3-DHP, followed by the cleavage of the pyridine ring to produce unnamed pyruvates that are non-toxic and may have positive nutritional qualities (Allison *et al.* 1992). Inoculation with *S. jonesii* enhanced the value of leucaena as an animal feed.

These researchers also reported that *S. jonesii* was not naturally present in ruminants and could be found in just a few restricted regions of the world where there had been a long history of leucaena feeding; notably in certain regions of Hawaii and Indonesia, and in some other tropical countries such as Thailand and Malaysia (Jones 1981; Jones and Lowry 1984), but not in Australia. Accordingly, the bacterium was introduced to experimental Australian steers via inoculation using a bacterial culture, originally sourced from rumen fluid from a Hawaiian goat in 1982 (Jones and Megarrity 1986). Following local testing and validation of the efficacy of the bacterium, from 1984 strained rumen fluid from cattle at CSIRO Lansdown Research Station near Townsville (Jones and Megarrity 1986), and Queensland Government Brian Pastures Research Station near Gayndah (Quirk *et al.* 1988) was used to inoculate Australian cattle herds grazing leucaena pastures. It was shown that a one-off inoculation of a small proportion of herds (*c*. 10%) using an oral drench was sufficient to provide long-term herd-wide protection provided: (a) the herd was maintained on leucaena pastures during the growing season, and; (b) that carrier animals were available to inoculate newly arrived naïve animals (Jones and Megarrity 1986).

Since 1995, the inoculum has been produced in a fermenter and distributed as a frozen enrichment culture of rumen microorganisms including *S. jonesii* (Klieve *et al.* 2002). These achievements led to increased adoption of leucaena pastures in Queensland to over 200,000 ha planted (Dalzell *et al.* 2012) with cattle achieving annual LWGs of up to 300 kg (Wildin 1994). At this point, the toxicity problem in Australia seemed to be resolved.

However, concerns were raised following the results of a 2004 survey, and extensive herd urine tests, which found that up to 50% of northern Australian cattle herds were excreting high levels of urinary DHP, despite most graziers having taken steps to ensure that their herds were protected (Dalzell *et al.* 2012; Graham *et al.* 2013). This finding was considered a serious impediment to productivity and was often not appreciated by graziers, as there were usually no visible clinical signs of toxicity, and there was an apparent benefit of leucaena fed for short periods at 30–60% of diet. It was hypothesised that the commercial fermenter culture containing *S. jonesii* inoculum may be less efficient than the original rumen fluid inoculum, thus reducing its ability to degrade DHP to levels below a suggested critical toxicity threshold (100 mg DHP/L) (Dalzell *et al.* 2012).

Accordingly, this study was initiated to investigate the efficacy of the fermenter cultured *S. jonesii* inoculum. A controlled animal house trial was conducted using a range of leucaena feeding regimes to monitor the onset of DHP toxicity and the impact of inoculation with the fermenter enrichment culture containing *S. jonesii*.

5.2 Materials and methods

5.2.1 Treatments and animal management

Sixteen one-year-old Charolais × Santa Gertrudis steers (*Bos indicus*) (average starting weight: 270 ± 26.2 kg (± s.d.)) were used in a 10-week controlled feeding trial, commencing in June 2010. The trial was conducted at a private research facility located at Mt Cotton, Brisbane, Queensland, Australia (27º 36' 29" S, 153º 14' 21" E). The trial consisted of a 6-week pre-inoculation feeding period, and a 4-week post-inoculation feeding period. Treatments were three combinations of forage-harvested *Leucaena leucocephala* cv. Tarramba (leucaena) and *Chloris gayana* (Rhodes grass) hay, namely: leucaena offered at 25%, 50% & 100% of the diet. A fourth treatment comprised 50% leucaena in period 1, switched to 50% *Medicago sativa* (lucerne) hay in period 2. The lucerne hay treatment was offered so that the rate of disappearance of DHP in leucaena fed animals following inoculation could be compared with disappearance when leucaena was removed from the diet. Total daily dry matter offered was 25 g DM/kg LW.day. Treatments were arranged in a randomised complete block design (RCBD) with 4 replications. At the commencement of period 2, all steers were inoculated with 100 mL of fermenter produced rumen enrichment culture containing *S. jonesii* as an oral drench (Klieve *et al.* 2002). The trial was sanctioned under animal ethics approval # LCAFS/035/10 (Appendix 2).

The forage-harvested and air-dried leucaena cv. Tarramba contained ~60% leaf matter and ~40% stem, w/w. Drying occurred for a period of 1 week, and DM% was ~91%. Due to supply issues, the Rhodes grass was not consistent between periods of the trial. The first batch, used in period 1 (pre-inoculation) was contaminated with lucerne whereas the second batch, used in period 2 (post-inoculation) had no lucerne contamination. Prior to commencement of period 1, there was a 10-day adaptation period on Rhodes grass (period 1 batch).

All animals used in this study had no previous exposure to leucaena. While the Mt Cotton research facility had a history of *S. jonesii* inoculated cattle grazing leucaena, the trial steers were housed separately in pens that were thoroughly cleaned and disinfected with antibacterial cleansers prior to their arrival.

5.2.2 Daily dry matter intake

Dry matter intake (DMI) of each feed-type was recorded daily, with mean weekly values calculated. Dry matter (DM) content of oven-dried (60°C) samples was determined by drying to a constant weight. The content of nitrogen (N) in samples was determined using a LECO TruSpec CHN analyser (Matejovic 1995), neutral detergent fibre (NDF) and acid detergent fibre (ADF) was measured according to the method described in Van Soest *et al.* (1991), and gross energy (GE) was determined by using an Auto Bomb Calorimeter LECO model AC-350 Corporation (USA) (Jetana *et al.* 2011).

5.2.3 Urinary DHP measurements

Weekly urine was obtained from each animal as a bulked 24-hour collection in a metabolism crate, with each replicate sampled on a separate day, from Tuesday to Friday.

Urine volume was recorded and a subsample preserved by acidification at a rate of 1:19 with concentrated hydrochloric acid (HCl—32%) (Dalzell *et al.* 2012). The preserved urine samples were later analysed for DHP concentration using high performance liquid chromatography (HPLC), as described by Dalzell *et al.* (2012) with a corrected mobile phase of 25 mM ammonium dihydrogen phosphate. Total amount of urinary DHP excreted per 24 hours was determined based on the urine volume over that period.

5.2.4 Blood

Weekly blood serum samples were collected via jugular venipuncture into evacuated tubes (Becton Dickinson Vacutainer systems). Tubes were immediately chilled, and after clot activation, centrifuged at 1300 × g for 10 minutes before transferring separated serum into polypropylene tubes and freezing at −80°C as described by Graham *et al.* (2013). Serum samples were analysed by use of radioimmunoassay using RIAK4 and RIAK5 kits (Ghosh *et al.* 2007).

5.2.5 Rumen fluid and DNA extraction

Weekly rumen fluid samples were collected from all animals throughout the trial, including during the preliminary feeding period, via an orogastric tube and hand operated vacuum pump, as described by Graham *et al.* (2013) and stored at −80ºC. DNA was extracted from all rumen fluid samples using the cetyltrimethylammonium bromide (CTAB) method Gagen *et al.* (2010). Samples (1 mL) were centrifuged (13000 × g for 5 min), and the supernatant was removed before DNA extraction. Pellets were homogenized with 200 mg of silica-zirconium beads (1:1 mixture of 0.1 and 1.0 mm beads; Biospec, Bartlesville, OK)

and 800 µL of CTAB buffer in a FastPrep^{TM}-24 homogeniser (MP Biomedicals Australia) at maximum speed (6.5 s/m) for 1 min, twice. Samples were incubated at 70ºC for 20 min and centrifuged at 15000 × g for 10 min and the supernatant was extracted with an equal volume of 25:24:1 phenol-chloroform-isoamyl alcohol (Sigma Aldrich, Australia). After centrifugation, the aqueous phase was used for DNA precipitation using isopropanol and ethanol. The DNA pellet was resuspended in elution buffer (10 mM Tris-HCl, pH 8.0) quantified using NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Australia) and stored at −80ºC until PCR analysis.

5.2.6 Detection of Synergistes jonesii *by primary and nested PCR*

The gDNA from trial weeks −1, 1, 2, 3, 5, 6, 7, 9 and 10 was diluted 1:10 to measure approximately 50–100 ng/µL concentrations. Earlier, during optimisation of polymerase chain reaction (PCR) amplification of *S. jonesii* from rumen fluid derived gDNA, many samples did not show a product despite 40 cycles of PCR. In order to increase the sensitivity of *S. jonesii* detection, a second round of PCR using internal primer binding sites, termed “nested PCR” was used with the product of the first (“primary”) PCR as the template. DNA extracted from the oral drench (fermenter inoculum) containing *S. jonesii*, from 2008 and 2012 year batches, obtained from the Queensland Department of Agriculture and Fisheries (QDAF-inoculum), was used as a positive control.

Primers were designed to amplify the 16S rRNA gene (16S rDNA) of the *S. jonesii* with ARB software (Ludwig *et al.* 2004) and multiple alignments from other closely related bacterial 16S sequences belonging to the *Synergistetes* phylum (Table 5.1). Of these primer sets, Sj_60F–Sj_1039R and Sj_60F–Sj_1004R were used for primary PCR amplification. Approximately 50–100 ng of gDNA was used for each 25 µL PCR using Platinum Taq (Life Technologies, Carlsbad, CA) with 2 mM $MgCl_2$, 200 µM each of dNTPs and 200 nM each of forward and reverse primers. A high annealing temperature of 63ºC (72ºC extension) and amplification for only 25 cycles of amplification was done to prevent spurious products from becoming targets for amplification in the second, nested PCR. An aliquot (2 µL) of the primary PCR was cleaned with enzymes Exonuclease I and Antarctic Phosphatase (New England Biolabs, Ipswich, MA) to remove excess primers and dNTPs at 37ºC for 20 min, followed by deactivation of these enzymes at 80ºC for 20 min. Nested PCR of the cleaned primary product was done utilising Sj_137F–Sj_1004R or Sj_193F–Sj_1004R primer pairs in a 25 µL PCR, as described above.

Table 5.1: *S. jonesii* 16S rDNA specific primer sets used for primary and nested PCRs extracted from rumen fluid samples collected from *Bos indicus* steers fed leucaena.

[A], Also used as nested primer; [B](Graham *et al.* 2013)

PCR primers (5'–3')	
Primary PCR	
Sj_60F	AGTCGAACGGGGATCATGT
Sj_1004R[A]	CCTCTCGATCTCTCTCAAGTAAC
Sj_1039R[B]	CCATGCAGCACCTGTTCTAC
Nested primers	
Sj_137F	AGTTACAGGGGGACAACGGA
Sj_193F[B]	TAAAAGGAGCGATCCGGTAACA

5.2.7 Sequencing of product from S. jonesii *nested PCR positive samples*

S. jonesii positive PCR products (2 µL) were cleaned up by Exonuclease I and Antarctic Phosphatase digestion, as described above, prior to sequencing with BigDye Terminator v3.1 using the kit Protocol (ABI, Life Technologies, Grand Island, NY). Sequencing (20 µL, 50 cycles) reactions were run with primer Sj_137F or Sj_193F for the forward and Sj_1004R primer (3 pmoles each) for the reverse sequences. The sequenced products were cleaned-up using the ABI protocol and run on the ABI 3130xl Prism Genetic Analyser. Multiple alignment of all sequences was done using Vector NTI's Advance 11 software (Invitrogen, Life Technologies, USA) AlignX module using Clustal W with *S. jonesii* ATCC 49833 (78-1) type strain 16S rDNA as the reference.

Sequence chromatograms were quality-clipped with the PreGap4 module of the Staden Package (http://staden.sourceforge.net/staden_home.html) and sequences manually edited to correct base-calling of 'N's with MEGA5 (Molecular Evolutionary Genetics Analysis software (Tamura *et al.* 2011) followed by collation of sequences in the FASTA format. Paired forward and reverse sequences from each sample were 'contiged' on the Vector NTI ContigExpress™ module to generate a single sequence from the 5' to 3' end of the *S. jonesii* positive PCR product. Multiple alignment of all sequences was done on Vector NTI AlignX module using Clustal W and compared with *S. jonesii* ATCC 49833 (78-1) type strain 16S DNA as the reference. Any consistent point-mutations (single nucleotide polymorphisms — SNPs) were tabulated.

5.2.8 Statistical analyses

Data were analysed using Microsoft Excel computer software (©2011, Microsoft, Redmond, WA, USA) and Minitab® statistical software 16 (©2010, Minitab Inc., State College, PA, USA). ANOVA GLM models for repeated measures using a spilt-plot design were used to analyse mean concentrations of urinary 3,4-DHP, 2,3-DHP and total DHP; and changes in DMI and LWG between treatments and periods. One-way ANOVA tests were further used to compare differences in total urinary DHP, LWG, and DMI data.

A one-way ANOVA test was used to test frequency of detection between treatments in *S. jonesii* PCR data. An LSD for serum thyroxine levels was determined using one-way ANOVA with pooled error term. The relationship between total urinary DHP and DMI was investigated using normal regression analysis.

Descriptive statistics were used to summarise DHP concentrations, chemical composition and gross energy of the feed types, DMI, serum thyroxine, and *S. jonesii* PCR results for each period.

A probability level of $P < 0.05$ was considered statistically significant, while a *P*-value between 0.05–0.10 was considered a moderate trend.

5.3 Results

5.3.1 Chemical composition

The crude protein (CP) content of the leucaena chaff was consistent for each period (80 g/kg DM for period 1, and 81 g/kg DM for period 2) (Table 5.2). Crude protein (CP) values were low due to the high content of stem in the forage-harvested material. The CP content of the Rhodes grass was different for each period (76 g/kg DM vs 22 g/kg DM) for periods 1 and 2, respectively, due to some contamination of the first batch with lucerne chaff.

Table 5.2: Chemical composition (g/kg DM) and gross energy (MJ/kg DM) of feed types offered to *Bos indicus* steers.

DMD: dry matter digestibility; GE: gross energy; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; period 1: 6-week pre-inoculation period; period 2: 4-week post-inoculation period; [A], Rhodes grass chaff contaminated with lucerne chaff

Parameter	Leucaena chaff		Rhodes grass chaff		Lucerne chaff
Period	1	2	1[A]	2	2
DMD (g/kg)	474 ± 5	466 ± 9	553 ± 1	525	660
GE (MJ/kg DM)	17.5	17.5	16.9	16.6	17
CP (g/kg DM)	80	81	76	22	197
NDF (g/kg DM)	650	650	724	807	528
ADF (total) (g/kg DM)	519	519	513	516	449

5.3.2 Dry matter intake

While animals were offered a total daily ration equal to 25 g DM/kg LW.day, all leucaena treatments resulted in an average total daily DMI of <20 g DM/kg LW.day (Figure 5.1).

The steers on the 25% leucaena treatment (n = 4) had an average intake of 19.1 ± 0.2 g DM/kg LW.day in period 1, decreasing to 17.8 ± 0.2 g DM/kg LW.day in period 2 (P < 0.00). Steers consumed an average of 91% of the daily leucaena chaff offered (Figure 5.1a), and 68% of Rhodes grass offered. Thus, the average actual intake of leucaena was 31% of diet in period 1 and 34% of diet in period 2. The initial 4 days after introduction to treatment diets were excluded from analysis in 2 animals that did not consume any leucaena.

Steers on the 50% leucaena treatment (*n* = 4) had an average intake of 18.8 ± 0.2 g DM/kg LW.day over both periods 1 and 2. Steers consumed an average of 60% of the leucaena, and 90% of the Rhodes grass offered, with no significant difference between periods 1 and 2 (Figure 5.1b) (*P* = 0.29). The average actual intake of leucaena was 39% of diet.

The steers on the 50% leucaena treatment during period 1, and switched to lucerne in period 2 (*n* = 4), had an average intake of 18.8 ± 0.2 g DM/kg LW.day in period 1 (Figure 5.1c). There was a marked increase in intake of legume and decreased intake of Rhodes grass in period 2 (*P* < 0.01) (20.9 ± 0.3 g DM/kg LW.day) with steers consuming 93% of lucerne and 74% of Rhodes grass offered (Figure 5.1c). The average intake of lucerne was 57% of diet during period 2. In the final week of the trial, animals on the lucerne treatment consumed a total of 23.0 g DM/kg LW.day, the highest of any treatment.

Steers on the 100% leucaena treatment (*n* = 4) consumed an average of 15.5 ± 0.2 g DM/kg LW.day in period 1 (Figure 5.1d) and 17.5 ± 0.2 g DM/kg LW.day in period 2. The increase in intake of leucaena after inoculation was small but significant (*P* < 0.01). There was evidence that intake increased linearly with declining excretion of DHP in urine over the 10 weeks of the experiment (Figure 5.2a).

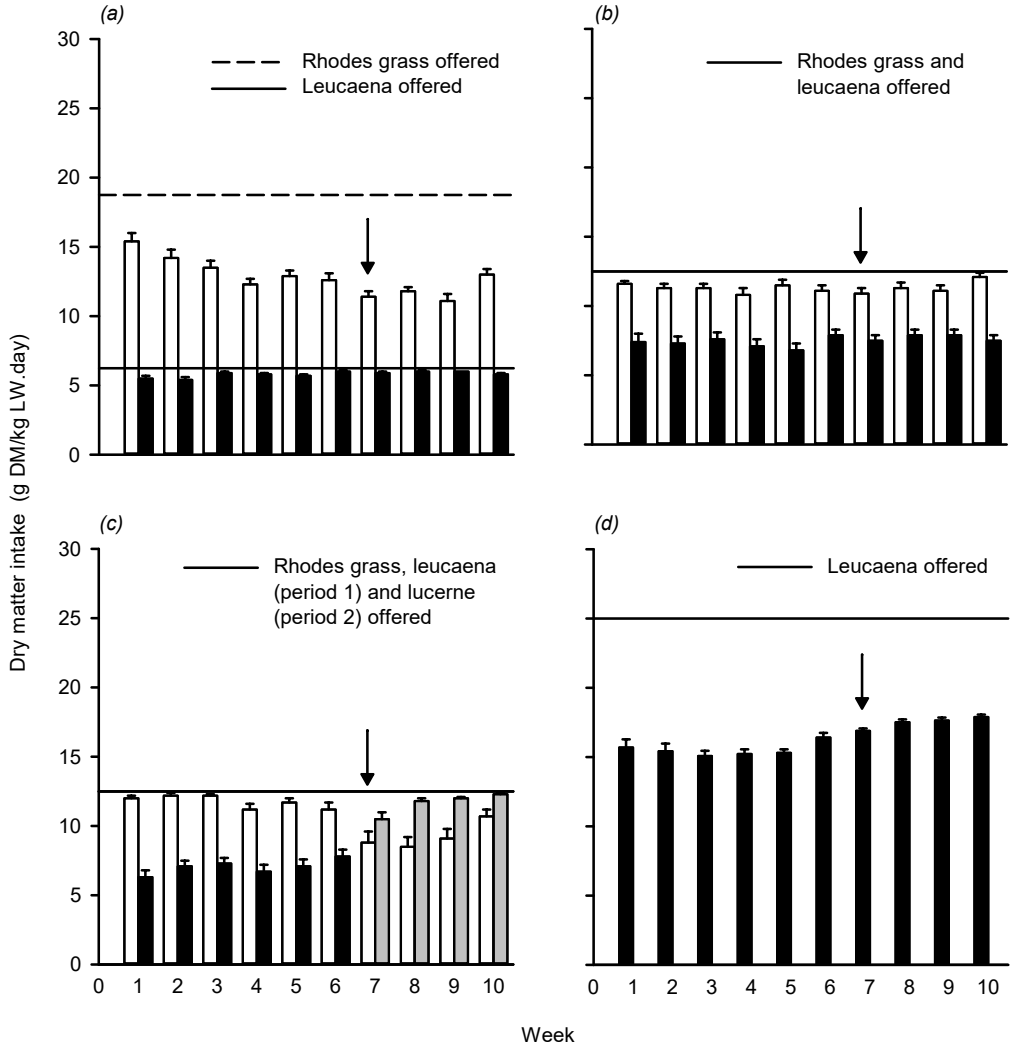

Figure 5.1: Mean weekly DMI (+s.e.) of Rhodes grass (□), leucaena (■), and lucerne (▩) for *Bos indicus* steers fed diets containing (a) 25% leucaena, (b) 50% leucaena, (c) 50% leucaena for period 1 & 50% lucerne for period 2, and (d) 100% leucaena. Arrows (⬇) indicate date of inoculation with cultured *S. jonesii*. DMI: dry matter intake; period 1: 6-week pre-inoculation period; period 2: 4-week post-inoculation period.

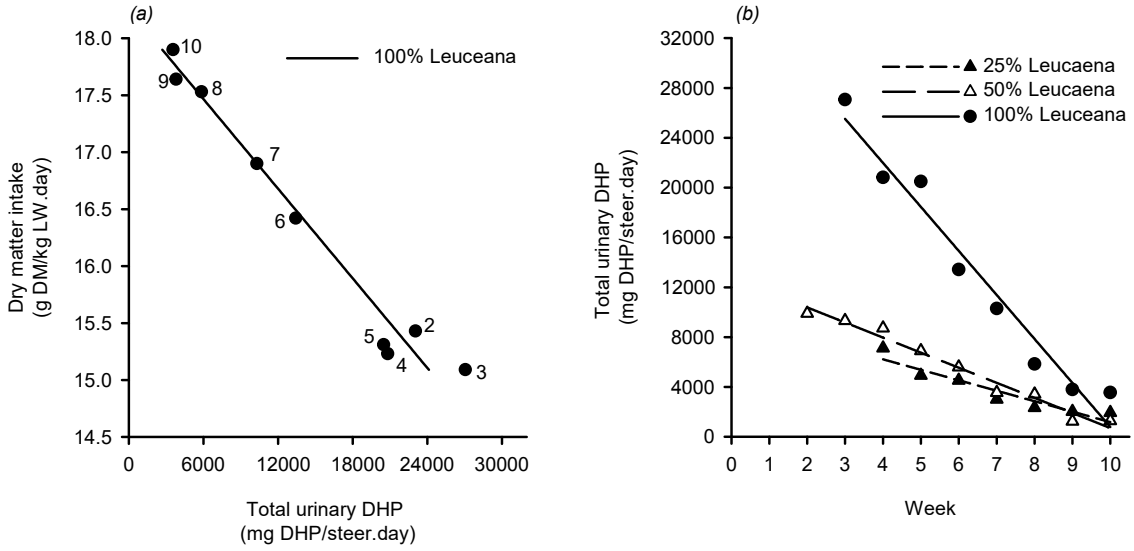

Figure 5.2: (a) Intake of leucaena (100% leucaena treatment only) in *Bos indicus* steers in response to decreasing urinary DHP excretion for weeks 2–10 (denoted as data labels) (DMI = -1.27×10^{-4} × total urinary DHP + 18.2; R^2 = 0.97, P < 0.001), and (b) rate of decline in mean total DHP excretion for steers fed diets containing 25% leucaena (total urinary DHP = −840 × week + 7062; R^2 = 0.89), 50% leucaena (total urinary DHP = −1211 × week + 11600; R^2 = 0.97) and 100% leucaena (total urinary DHP = −3533 × week + 29056; R^2 = 0.96)(P < 0.001). DHP: hydroxypyridone; DMI: dry matter intake; LW: liveweight.

5.3.3 Selection index

A feed selection index (SI) was calculated for the 25 and 50% leucaena, and the 50% lucerne (weeks 7–10) treatments (Table 5.3).

Selection index was defined by:

$$SI = \frac{fraction\ of\ feed\ consumed}{fraction\ of\ feed\ offered}$$

A SI of 1 indicated equal preference for legume and Rhodes grass, while a SI <1 indicated negative selection for that species.

In the 25% leucaena treatment (n = 4), there was a preference for leucaena over Rhodes from week 2 onwards, with almost complete consumption of all leucaena offered; the selection index for leucaena ranged between 1.09 and 1.41.

In all steers on the 50% leucaena treatment during period 1 (n = 8), there was a negative preference for leucaena with SI varying from 0.68 and 0.82. The SI for Rhodes grass remained between 1.17 and 1.32 throughout period 1, and continued unchanged for steers remaining on the 50% treatment during period 2 (n = 4). Selection index for Rhodes grass

however declined to 0.88 in the steers switched to lucerne during period 2 (*n* = 4); the mean SI for lucerne was 1.12.

Table 5.3: Mean selection index for period 1 and 2, for each treatment level of leucaena offered to *Bos indicus* steers.

Period 1: 6-week pre-inoculation period; period 2: 4-week post-inoculation period; [A], Rhodes grass chaff contaminated with lucerne chaff

Treatment	Period	n	Leucaena chaff	Rhodes grass chaff	Lucerne chaff
25% leucaena	1	4	1.22	0.93[A]	
	2	4	1.34	0.89	
50% leucaena	1	8	0.76	1.24[A]	
	2	4	0.81	1.19	
50% lucerne	2	4		0.88	1.12

5.3.4 Rumen microbial analysis

Of the 96 rumen fluid samples from period 1, ~22% were positive for *S. jonesii* 16S rDNA-based nested PCR; while from the 48 samples from period 2, ~21% were positive for *S. jonesii* (Table 5.4). Interestingly, of the samples collected before treatments began (week −1), 3 of 16 animals (~19%) were positive for *S. jonesii* despite never having previously encountered leucaena or been inoculated with *S. jonesii*. After leucaena treatments commenced, there was inconsistency in the number of samples positive for *S. jonesii*. In weeks 1, 2, 3, 5, 6, 7, 9, and 10 the number of positive rumen fluid samples were 19%, 25%, 13%, 38%, 19%, 31%, 19%, and 13%, respectively.

Of the 16 animals in the trial, 13 (~81%) were positive for *S. jonesii* at some stage by nested PCR either in period 1 (~69%) or period 2 (50%). Five animals (~31%) were positive for *S. jonesii* only in period 1 whereas only 2 (~13%) became positive for *S. jonesii* after inoculation (period 2); 6 animals (~38%) were positive for *S. jonesii* in both periods 1 & 2.

Multiple alignment analysis of the sequences against the type strain (ATCC 49833 (78.1) 16S rDNA for *S. jonesii* positive samples, revealed 4 loci with point mutations corresponding to *E. coli* 16S rDNA base positions 268(C→T), 306(A→G), 328(G→A) and 870(A→C). These SNPs when present occurred predominantly at loci 306 (100%) and 870

(~92%); to a lesser extent at locus 328 (~27%); and rarely at position 268 (~8%). Only ~16% of the 31 samples positive for *S. jonesii* were identical to the 16S rDNA sequence of type strain 78-1; 2 were from period 1, and 3 from period 2. DNA from the 2 batches of the QDAF fermenter culture oral-inoculum (years 2008 & 2012) both showed SNPs, but only at loci 306 and 870.

5.3.5 Urinary DHP excretion

DHP levels in urine varied with amount of leucaena in diet but were highest in the weeks immediately following commencement of leucaena feeding and continued to increase until weeks 3 and 4 (Figure 5.3). Thereafter, levels gradually declined. When all animals were inoculated at the end of week 6 (end of period 1) DHP levels continued to decline with no departure from linearity, indicating no effect of inoculation on rate of change of total DHP excretion (Figure 5.2b). Steers fed lucerne ceased all toxin excretion by week 8, 14 days after removal of leucaena from their diets (Figure 5.3c).

All treatment groups showed an increasing proportion of the isomer 2,3-DHP excreted during period 1, which then declined from week 7 to low levels during period 2 following inoculation (Figure 5.3). However, total DHP remained at levels greater than the suggested critical threshold of 350 mg/steer.day (100 mg/L, adapted from Dalzell *et al.* (2012) with an average daily urine volume of ~3.5 L) through to week 10, except when leucaena was replaced by lucerne.

Table 5.4: The number of times rumen fluid collected from *Bos indicus* steers tested positive for *S. jonesii* in nested PCR with SNPs detected in at least 1 loci; weeks 0,4 & 8 were not analysed.

Period 1: 6-week pre-inoculation period; period 2: 4-week post-inoculation period; SNPs: single nucleotide polymorphisms; [A], no SNPs detected in these samples; –, indicates *S. jonesii* was not detected

Treatment	Animal ID (*n* = 16)	Weeks in which animals were *S. jonesii* positive (SNPs were also detected)	
		Period 1 (*n* = 96)	Period 2 (*n* = 48)
25% leucaena	1	–	–
	2	3	–
	3	3,5	7,9,10
	4	1	–
50% leucaena	5	2[A]	10
	6	−1,1,2,5,6	–
	7	−1,2	7[A]
	8	−1,1,5,6	7
50% leucaena (period 1) & 50% lucerne (period 2)	13	–	–
	10	5	9[A]
	11	–	9
	12	5,6[A]	–
100% leucaena	13	5	–
	14	–	–
	15	–	7[A]
	16	2	7

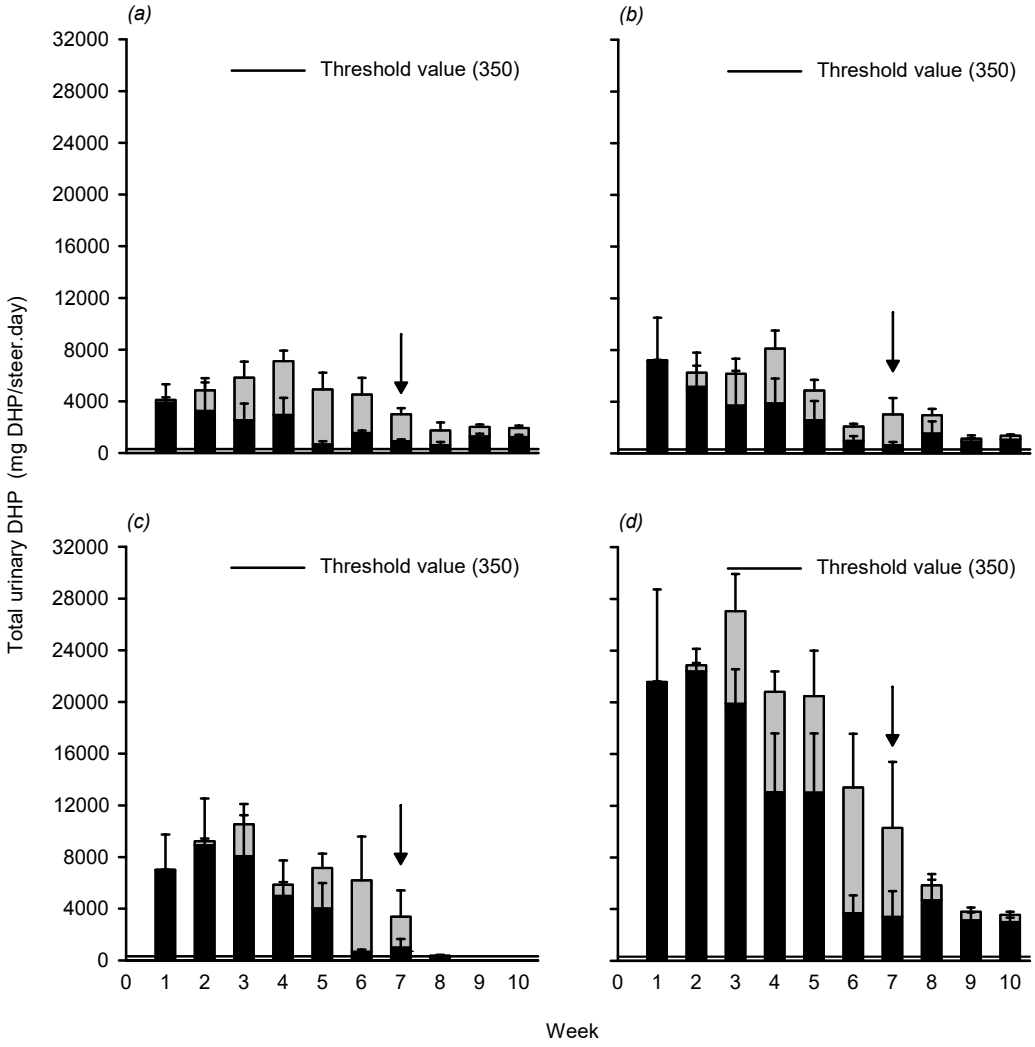

Figure 5.3: Mean weekly DHP excretion patterns of 3,4-DHP (■) and 2,3-DHP (▩) for *Bos indicus* steers fed diets containing: (a) 25% leucaena, (b) 50% leucaena, (c) 50% leucaena for period 1 & 50% lucerne for period 2, and (d) 100% leucaena (+s.e.). Arrows (⬇) indicate date of inoculation with cultured *S. jonesii*. DHP: hydroxypyridone; period 1: 6-week pre-inoculation period; period 2: 4-week post-inoculation period.

5.3.6 Serum thyroxine

Serum thyroxine (T_4) levels (Figure 5.4) decreased rapidly during the preliminary period prior to commencement of leucaena feeding (during week −1) and were lowest on the first day of leucaena feeding when concentrations were 54, 43, and 33 nmol/L for animals receiving treatments of 25%, 50%, and 100% leucaena, respectively. Concentrations subsequently increased to levels within the normal range (70–120 nmol/L) (Jones *et al.* 1978) and remained within this range for the remainder of trial. Animals receiving 25% leucaena exhibited higher serum T_4 concentrations than those receiving 100% leucaena during period 1. No significant difference was evident among treatments during period 2.

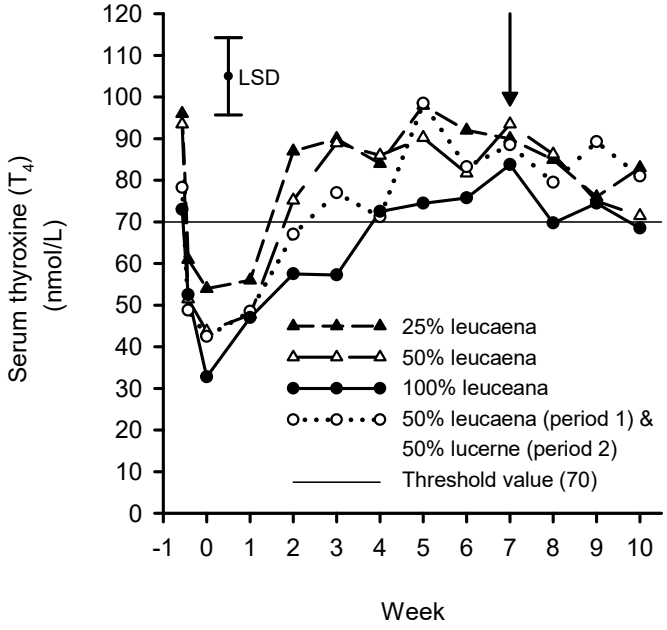

Figure 5.4: Mean serum thyroxine (T_4) concentration for *Bos indicus* steers fed diets containing 25% leucaena, 50% leucaena, 50% leucaena for period 1 & 50% lucerne for period 2, and 100% leucaena. Bar indicates 1 × $LSD_{0.05}$ where difference in T_4 levels was significant ($P < 0.05$). Arrow (⬇) indicates date of inoculation with cultured *S. jonesii*. Period 1: 6-week pre-inoculation period; period 2: 4-week post-inoculation period.

5.4 Discussion

There were several important findings from this trial, many of which contradicted the original theories described for leucaena toxicity. These original understandings and the findings are now compared and contrasted.

5.4.1 Efficacy of inoculation with cultured S. jonesii

In past work, inoculation with rumen fluid containing *S. jonesii* was shown to lead to rapid degradation of DHP and therefore provided very efficient protection from toxicity (Jones and Megarrity 1983). In this trial, total DHP began to decline prior to inoculation and there was no change in the rate of decline after inoculation with cultured *S. jonesii* (Figure 5.2b) suggesting no discernible effect of the inoculum on total DHP excretion. Furthermore, urinary DHP results indicated that the inoculum was not capable of reducing total DHP levels to below the suggested critical threshold for toxicity (350 mg/steer.day adapted from Dalzell *et al.* (2012)) within 4 weeks of inoculation.

These results contrast the original work on rumen inoculation, which found that *S. jonesii* completely detoxified all DHP within 5-days after inoculation using *in vivo* sourced rumen fluid (Jones and Lowry 1984; Jones and Megarrity 1986). In other studies using *in vivo* sourced *S. jonesii,* Quirk *et al.* (1988) and Pratchett *et al.* (1991) reported that inoculated steers grazing leucaena ceased DHP excretion within 6 and 4 weeks, respectively.

5.4.2 High levels of 2,3-DHP

The compound 3,4-DHP was originally reported to be the principal isomer excreted in unprotected animals, with 2,3-DHP perceived as transitory. Indeed 2,3-DHP was thought to be present only temporarily as the isomerization product of 3,4-DHP prior to degradation of the pyridine ring by *S. jonesii* in newly inoculated animals (Jones *et al.* 2009). In this trial, after 6 weeks consuming leucaena and prior to inoculation, 2,3-DHP levels were present in higher proportions than 3,4-DHP (Figure 5.3). The high levels of 2,3-DHP excreted in urine were in accordance with the findings of much recent work. In the grazier survey in Queensland, Dalzell *et al.* (2012) showed that almost half of the 44 herds tested had levels of urinary DHP above 100 mg/L and over half of these were excreting higher proportions of 2,3-DHP than 3,4-DHP. Other workers Ghosh *et al.* (2007); Palmer *et al.* (2010); Phaikaew *et al.* (2012); Graham *et al.* (2013) also reported high levels of 2,3-DHP in ruminants after periods of between 2–10 weeks from commencement of consuming

leucaena. These findings, and those of the current study, strongly contradict the notion that 2,3-DHP is a transitory isomer.

In the current experiment, 2,3-DHP began to appear in urine samples from week 2 and became the predominant isomer by week 6. The accumulation of 2,3-DHP occurred simultaneously with a decline in 3,4-DHP, and therefore total DHP. These findings suggest the presence of an indigenous strain of *S. jonesii*, which converted 3,4-DHP to 2,3-DHP at a faster rate than 2,3-DHP was able to be degraded. Following inoculation, 2,3-DHP excretion was reduced relative to 3,4-DHP indicating that the inoculum may have been more effective in degrading 2,3-DHP and that metabolism of each of these isomers is controlled by different regulatory conditions which may vary between strains of *S. jonesii* (Hammond *et al.* 1989; Rincón *et al.* 2000).

Whilst not as widely researched as 3,4-DHP, the isomer 2,3-DHP is also considered a potent goitrogen (D'Mello 1992; Paul 2000) and has been shown to have acute toxic effects when directly infused intraruminally (McSweeney *et al.* 1984; Puchała *et al.* 1995). For these reasons, in the assessment of leucaena toxicosis, total DHP excretion was considered.

5.4.3 Detection of S. jonesii *prior to inoculation*

Synergistes jonesii has been reported to be a unique bacterium present in specific countries and at specific locations. However, in this trial, PCR analysis of rumen fluid from steers naïve to leucaena, from a coastal property where *S. jonesii* had not been introduced, detected the presence of *S. jonesii*, albeit at low numbers ($<10^4$ cells per mL). Thus, while DHP excretion in all treatments was initially quite high, this indigenous population of *S. jonesii* was most likely responsible for the gradual decline in urinary DHP levels observed in all leucaena diets prior to inoculation. A decline in urinary DHP in uninoculated ruminants was also observed in the work of Ghosh *et al.* (2007) and Graham *et al.* (2013).

As *S. jonesii* is difficult to detect in rumen fluid of livestock, the presence of *S. jonesii* has previously been inferred by either a colorimetric analysis of DHP in urine (Jones and Megarrity 1983; Lowry *et al.* 1985; Jones 1994) and/or by HPLC analysis of urine (Tangendjaja and Wills 1980; Dalzell *et al.* 2012), or by detecting mimosine and DHP degradation by a rumen culture enrichment (Allison *et al.* 1990; Allison *et al.* 1992). Each of these methods has merits and drawbacks, however they do not directly detect *S. jonesii*.

Graham *et al.* (2013) reported the first use of a nested PCR approach for direct *S. jonesii* detection. They found that detection was sporadic due to very low populations of the bacterium in the rumen. The current study again employed a nested PCR approach using novel *S. jonesii* specific primer pairs for both the primary and nested PCRs; in the previous study, Graham *et al.* (2013) used a *S. jonesii*-specific forward primer with a general bacterial reverse primer (1275R). The current approach was able to detect *S. jonesii* in ~22% of all samples collected; almost 4 times more frequently than in the study of Graham *et al.* (2013). This increased sensitivity enabled detection of *S. jonesii* in most steers at least once across the 10 weeks of the trial. The advancement of more specific primers is required to further overcome the sporadic detection, as evidenced in this trial.

Other surveys of *S. jonesii* in ruminant species (cattle, goats, sheep, buffalo, yak and native Indonesian and Jinnan cattle) from several countries (Australia, Indonesia, Thailand, China, Vietnam and Brazil) used a similar nested PCR-based approach (Padmanabha *et al.* 2014). Sequence confirmation of *S. jonesii* from these studies now suggest ubiquity of *S. jonesii* in the rumen of different animal species from diverse parts of the globe (Padmanabha *et al.* 2014). This contradicts the notion that *S. jonesii* is unique only to ruminants in certain countries.

However, in all surveys, the infrequent detection of *S. jonesii* indicated that abundance was low and probably often below the limits of sensitivity/detection by 16S rDNA-based PCR assays. The limit of detection by PCR/nested-PCR is theoretically determined to be ~3 × 10^3 *S. jonesii* cells/mL for pure cultures and <10^4–10^5 cells/mL for rumen digesta (Graham *et al.* 2013).

Whilst there is evidence *S. jonesii* is indigenous to the rumen, positive PCR detection does not infer functional capacity to degrade DHP (McSweeney and Shelton 2009). It is also possible that the introduced strains of *S. jonesii* have become pandemic in Australian ruminants since its release in the 1980s (Jones *et al.* 1985b) as *S. jonesii* can easily spread among uninoculated animals within a herd (Klieve *et al.* 2002) and to nearby uninoculated herds if handling facilities are in common (Quirk *et al.* 1988; Pratchett *et al.* 1991). However, the frequent detection internationally likely suggests that *S. jonesii* is indeed normal rumen flora.

It has been previously thought that the persistence and abundance of *S. jonesii* is dependent upon the presence of leucaena in the diet (Rincón *et al.* 2000). However, in the current study this relationship was not obvious. There was no clear trend of greater frequency of *S. jonesii* positive samples with (a) length of time animals were consuming

leucaena, (b) cattle fed 100% compared to 25% and 50% leucaena in diet, or (c) numbers of animals positive for *S. jonesii* pre- and post-oral inoculation. Padmanabha *et al.* (2014) also found that a leucaena diet, or lack thereof, did not influence detection of *S. jonesii* in samples from ruminants in several countries. Similar trends were also observed in the field studies of Graham *et al.* (2013). As *S. jonesii* is a chemoorganotroph (Allison *et al.* 1992) it relies on the breaking of chemical bonds of amino acids for energy and grows best with arginine and histidine as substrates (Allison *et al.* 1992; Rincón *et al.* 1998), which gives it a competitive physiological niche in the rumen (McSweeney *et al.* 1993a). By utilising these, as well as other amino acids and peptides, *S. jonesii* is capable of survival in the absence of mimosine and DHP in diet (McSweeney *et al.* 2002). Indeed *S. jonesii* does not require DHP for growth *in vitro* (McSweeney *et al.* 1993a). Hammond *et al.* (1989) also reported that three years after removal from the island of St Croix, and without any leucaena in diet, cows retained the capability to degrade 2,3-DHP.

5.4.4 Presence and function of different strains of S. jonesii

The latest techniques utilizing nested PCR detection methodology have given new insight into the genetic diversity of *S. jonesii*. The frequent detection of SNPs at various loci in both the cultured inoculum and the indigenous *S. jonesii* population in the steers suggests there are multiple strains of *S. jonesii* present in animals and the inoculum, which probably differ genetically and functionally from the original isolates and the type strain 78-1 (ATCC 49833), with which most culture studies have been performed. The type strain and the additional three labelled strains (100-6, 113-4 and 147-1) were originally isolated from *in vitro* serial transfers of the original source of rumen fluid from a Hawaiian goat and all shared an identical 16S rDNA sequence (Allison *et al.* 1992). While all strains were able to degrade 3,4-DHP and 2,3-DHP, and were phylogenetically identical, some were selectively more efficient at degrading a particular isomer of DHP; for example, 78-1 effectively degrades 3,4-DHP (McSweeney and Shelton 2009). Although the 4 strains were isolated from samples from the same Hawaiian goat used to source the inoculum introduced to Australian cattle in 1982, the Australian inoculum was composed of 2 unnamed strains, isolated independently from separate enrichments (Jones 1994), for which the DHP isomer-specificity is unknown.

Domínguez-Bello *et al.* (1997) reported that degradation of 2,3-DHP, the final step before pyridine ring cleavage, began to occur when *S. jonesii* was present in high numbers. This was also regulated by the concentration of pyridinediols present to activate the substrate-induced isomerase enzymes for degradation to take place (Rincón *et al.* 2000). As

previously discussed, the number of cells of *S. jonesii* was very low in this trial and this may be a contributing factor to its low efficacy. As such, degradation may be delayed until sufficient proliferation of the bacterium (Hammond *et al.* 1989), which was not observed.

While *S. jonesii* is the only bacterium in culture collection that can degrade both isomers of DHP, it should be noted that Hammond *et al.* (1989) isolated rumen bacteria from cows that were not *S. jonesii* but were capable of degrading 2,3-DHP but not 3,4-DHP. Tan *et al.* (1994) reportedly isolated 4 strains of bacteria capable of degrading mimosine, 3,4-DHP and/or 2,3-DHP—*Lactobacillus spp.*, *Streptococcus bovis* and *Clostridium sporogenes*—which together were able to degrade up to 60% of DHP *in vitro* within 3 days. Domínguez-Bello and Stewart (1990) isolated 18 cultures which could degrade either 3,4-DHP or 2,3-DHP from a sheep in Venezuela naïve to leucaena, including gram-positive, and gram-negative bacteria and a gram-positive spore-forming bacterium (Domínguez-Bello and Stewart 1991). The work of Aung *et al.* (2011) isolated an aero-tolerant gram-negative *Coccobacillus* species able to degrade DHP, belonging to the genus *Klebsiella*, in ruminants naïve to leucaena. Additional bacteria include another *Streptococci sp.*, (Chhabra *et al.* 1998), a *Clostridium* (Domínguez-Bello *et al.* 1997), *Streptococcus lutetiensis*, *Clostridium butyricum*, *Lactobacillus vitulinus*, and *Butyrivibrio fibrisolvens* (Derakhshani *et al.* 2016). While, these observations cannot be verified as none of these isolates currently exist in culture collections it nevertheless suggests that it is quite possible that a consortium of many morphological types of bacterial species and/or strains is involved in the complete degradation of 3,4-DHP (Domínguez-Bello and Stewart 1990; McSweeney *et al.* 2002; McSweeney and Shelton 2009).

Molecular analysis of *S. jonesii* in this study detected discrete mutations (SNPs) in almost 84% of positive samples, which occurred consistently at four loci (268, 306, 328 & 870) in the 16S rRNA gene. As loci 306 and 870 attracted considerably more SNPs than loci 328 and 268, it suggested a possible hierarchy of propensity to attract mutations. The same SNPs at these two most prominent loci were also recorded for the QDAF oral-inoculum from two different batches produced 6-years apart.

However, all sequences were >97% identical to the *S. jonesii* ATCC strain 78-1. A similar trend for the same loci to acquire SNPs was evidenced in the survey of ruminants from Australia and other countries (Padmanabha *et al.* 2014). The presence of SNPs in the 16S rRNA gene can be a harbinger of a break from the type strain and the development of a new ‘strain’ for *S. jonesii*, which could differentially affect its ability to degrade DHP isomers.

It is clear now that Australian cattle host strains of *S. jonesii* that are different from the type strain 78-1 and the other strains originally isolated from the Hawaiian goat. These bacteria appear to persist in the rumen without the presence of leucaena in the animals' diet. The inconsistency in detection of *S. jonesii* by the current PCR test indicated that a more sensitive amplification-based detection is warranted.

5.4.5 Dry matter intake & selection index

The Rhodes grass offered during period 1 was contaminated with lucerne chaff, and consequently had a higher crude protein content in period 1 compared to period 2. It was therefore possible that the lack of difference in intake and selection index for 25% and 50% leucaena treatments was partially due to the higher quality of the Rhodes grass in period 1. However, this issue was not applicable to the 100% leucaena treatment. Previous studies reported that intake of leucaena and LWG increased following inoculation (Jones and Lowry 1984; Jones and Megarrity 1986; Quirk *et al.* 1988). A slight increase was partially observed in this trial only for the 100% leucaena diet, further suggesting a lack of (complete) protection afforded by the inoculum.

Fresh leucaena is a high-quality and highly palatable legume feed (Jones 1979; Andrew *et al.* 2000) and in the absence of DHP toxicosis, intake should be high (Dixon and Coates 2008). However, throughout the experiment, leucaena chaff DMI remained below that required for a high production diet (Gupta and Atreja 1999; Dixon and Coates 2008).

The original research reported that goats fed 100% leucaena for 5 weeks prior to inoculation had a stable but low daily intake, which doubled within 3 days after transfer of *in vivo* rumen fluid (Jones and Lowry 1984). A significant increase in intake of leucaena (and LWG) post-inoculation was also noted by Pratchett *et al.* (1991) in a grazing trial using irrigated leucaena.

In this study, there was a strong relationship between DHP excretion and intake in the 100% leucaena treatment (Figure 5.2a) suggesting that suppression of intake in the initial weeks could be attributed to high levels of circulating DHP. Interpreting this relationship further, maximum intake would have reached 18.2 g DM/kg LW.day when zero DHP excretion was reached i.e. complete degradation. An intake of this level would not constitute a high production diet, and intake was probably constrained due to the high stem content of the leucaena chaff that reduced the crude protein content (~8% DM) and digestibility (<48% DMD) of the chaff. At higher levels of leucaena offered, stem was often selected against, while animals consuming lower levels of leucaena readily consumed all

stem material. Regardless of stem content, the refusals from steers fed 100% leucaena always contained uneaten leaf material in addition to uneaten stem, suggesting that feed quality alone was not solely responsible for the reduced intakes. No similar relationships between intake and DHP excretion were observed in animals offered 25% or 50% leucaena in this trial. Since the actual intakes for these treatments were 31% and 39%, they were unlikely to cause significant intake suppression over the short period of time of this trial (Jones and Hegarty 1984).

Leucaena was preferred over Rhodes grass when fed at the lowest level of 25% and was completely consumed with a positive selection index (Table 5.3). However, there was clear selection against leucaena when offered at 50 or 100%, leading to reduced intake. When the 50% leucaena diet was switched to a 50% lucerne diet, intake of legume increased (Figure 5.1c), reflecting the much higher selection index of lucerne compared to leucaena (Table 5.3). Although fresh leucaena has been reported to be of comparable in quality to that of lucerne (Shelton and Brewbaker 1994) the forage-harvested leucaena chaff that contained stem was clearly less preferred. Ghosh *et al.* (2007) used a similar feed and also recorded a reduction in leucaena intake in unprotected animals as levels of leucaena offered increased. When protected from toxicity, cattle will readily select diets high in leucaena (Jones and Jones 1984), and can safely tolerate 100% leucaena diets (Halliday *et al.* 2014b). Pratchett *et al.* (1991) also reported high leucaena diets (70–95%) were consumed by shorthorn heifers after they acquired *S. jonesii*. Dixon and Coates (2008) used faecal near infrared reflectance spectroscopy (F.NIRS) to measure amount of legume in the diet of steers free-grazing leucaena/grass pastures and found that intake levels were as high as 30 g DM/kg LW.day, and were regularly in the range of 20–25 g DM/kg LW.day.

The intake results from this experiment confirmed the original notion that short-term feeding of leucaena below 30% of diet is considered ‘safe’ (Jones and Hegarty 1984), with few of the deleterious impacts on production that are often observed at higher levels of feeding (e.g. reduced intake and decreased LWG).

5.4.6 Serum thyroxine

Earlier work indicated that DHP was a potent goitrogen that could strongly suppress T_4 in response to toxicity (Hegarty *et al.* 1979), reportedly due to the inhibition of the iodination of tyrosine-based hormones due to its analogous nature with pyridoxal which antagonises peroxidase-based enzyme pathways (Christie *et al.* 1979; Lee *et al.* 1980). Thyroxine

levels were found to return to normal after removal of leucaena from diet or inoculation with *S. jonesii* (Jones *et al.* 1978; Megarrity and Jones 1983; Ghosh *et al.* 2007). The findings of this trial were in contrast as T4 levels were low even before the consumption of leucaena, and generally increased with time on leucaena. T_4 concentrations remained within the normal range (70–120 nmol/L) for animals on 25% and 50% treatments after 2 weeks on leucaena. Animals on 100% leucaena experienced lower levels of T_4 during period 1 but returned to the normal range after 4 weeks.

It is possible that alternate coping mechanisms, for instance conjugation, were involved in preventing the goitrogenic effects of DHP from manifesting. Although the level of conjugated DHP was not measured, with urine samples hydrolysed prior to HPLC analysis, as much as 70% of DHP in circulation has been reported as conjugated (Hegarty *et al.* 1979). Hepatic conjugation of xenobiotics is a common phase II detoxification pathway which can greatly reduce the bioactivity of toxins (Lindsay *et al.* 1974; Christie *et al.* 1979). The detoxifying effect of conjugation is due to the attachment of an acidic molecule at the active binding hydroxyl site of DHP, preventing any antagonistic or chelatory effects (Lowry *et al.* 1985).

5.4.7 Signs of toxicity

None of the animals, even consuming 100% leucaena, displayed clinical signs of leucaena toxicity during the trial, e.g. short-term hair loss, despite high levels of DHP recorded. Subclinical toxicity, such as reduced intake and LWG, may have occurred, however these results are confounded with feed quality in the experiment. Signs of subclinical toxicity however, have been reported in animals fed leucaena for longer periods (Jones *et al.* 1976; Quirk *et al.* 1988; Phaikaew *et al.* 2012) with Pratchett *et al.* (1991) reporting a LWG of only 0.40 kg/day in heifers grazing 70–95% leucaena over a 20-week period. Other work has reported unprotected steers fed 100% leucaena gained as little as 0.18 kg/day, with 2 animals dying (Jones *et al.* 1978).

In stark contrast to the above feeding trials, there is evidence of uninoculated cattle achieving high LWG on high leucaena diets, despite high urinary DHP levels. Bali bulls (*Bos javanicus*) in Indonesia on 100% leucaena diets were reported to be gaining weight near to their maximum genetic potential for the breed of 0.85 kg/steer.day (Mastika 2003; Halliday *et al.* 2014b; Panjaitan *et al.* 2014). Unexpectedly, these animals were shown to be excreting high levels of DHP in urine, demonstrating that they may possess an

alternative mode of adaptation to leucaena toxicity. It is hypothesized that conjugation of the toxin may be the operative pathway (Elliott *et al.* 1985); if so, this represents a major shift in the way leucaena toxicity could be managed. Further work is required to investigate the extent and significance of this detoxification pathway in Australian cattle.

5.5 Conclusions

This study demonstrated that the cultured *S. jonesii* inoculum had reduced capacity to protect animals on high leucaena diets from toxicity compared to the earlier research in which inoculation with rumen fluid from protected animals completely and rapidly degraded DHP.

Inoculation had no effect on the rate of degradation of total DHP and the levels of urinary DHP remained well above that considered to be a safe threshold. Despite this, thyroxine levels were not significantly reduced. The results contradicted the long-standing belief that 2,3-DHP is a transitory isomer. There was a small increase in intake of animals on 100% leucaena post-inoculation, but overall feed intake levels remained low.

It was found that an indigenous population of *S. jonesii* was present in the rumen of steers prior to inoculation. There was evidence of different strains (detected as SNPs) of *S. jonesii*, in both the indigenous population and the cultured inoculum, that were different to the type strain 78-1. These strains may have differential capacity to degrade the two DHP isomers thus contributing to varying proportions of 3,4-DHP and 2,3-DHP detected in urine before and after inoculation.

While not measured or discussed in this study, further research should investigate the possible effects of conjugation and chelation of DHP as an alternative means of limiting leucaena toxicity in ruminants worldwide and in Australian beef cattle.

Chapter 6 Survey of toxicity status of ruminants consuming *Leucaena leucocephala* in eastern Indonesia

6.1 Introduction

The productive forage tree legume *Leucaena leucocephala* (leucaena) is traditionally used as a feed for ruminant livestock in cut-and-carry systems in eastern Indonesia (Piggin and Parera 1985) where its high nutritive value and protein content make it ideal for cattle fattening, especially through the extended dry season. Although highly nutritious, leucaena contains the toxic non-protein free amino acid mimosine in relatively high concentrations (4–9% dry matter (DM) in young leaves and green seeds), which can adversely affect animal production (Hegarty *et al.* 1976).

In ruminants, the degradation of mimosine to 3-hydroxy-4(1*H*)-pyridone (3,4-DHP) post-ingestion occurs via many endogenous rumen bacteria (Hegarty *et al.* 1964b) and plant hydrolase enzymes within leaves (Lowry *et al.* 1983). The isomerisation of 3,4-DHP to 3-hydroxy-2(1*H*)-pyridone (2,3-DHP) and further catabolism of the pyridine ring, however, has been reported to require the specialised rumen bacterium *Synergistes jonesii* (Jones and Megarrity 1983; Allison *et al.* 1992), the presence of which was thought to be limited to specific tropical countries where leucaena feeding was historically practiced (Jones 1994). *S. jonesii* was originally discovered in Hawaiian goats, and later in Indonesian goats, that were consuming leucaena without ill affect (Jones 1981; Jones and Megarrity 1986), and was shown to be capable of detoxification of 3,4-DHP and 2,3-DHP to non-toxic by-products (Jones and Lowry 1984).

This led to the assumption that all ruminants in those countries where *S. jonesii* occurred naturally were protected from DHP toxicity (Jones 1994). However, in eastern Indonesia, where leucaena had become naturalised, adoption of leucaena-based cattle fattening systems was sporadic, with the occurrence of signs of DHP toxicity reported to be a potential barrier to adoption. It was hypothesised that in remote regions and islands, where animal movement is restricted, the spread of the bacterium may be impeded, and that animals may not be protected from DHP toxicity by *S. jonesii*.

In chapter 4, a field study to test the effectiveness of transfer of rumen fluid from ‘apparently protected’ animals to ‘apparently unprotected’ animals did not show any positive effect of inoculation. It was found that *S. jonesii* occurred at low populations in the rumen, and that there may be variation in the functional capacity of *S. jonesii*.

Accordingly, a field study was initiated with the objective of surveying the toxicity status of ruminants consuming leucaena. As leucaena-based cattle fattening presents an excellent opportunity to improve the livelihoods of smallholder farmers, overcoming possible toxicity limitations could enhance adoption and result in substantial benefits to rural communities.

6.2 Materials and Methods

6.2.1 Animal selection

A study of the occurrence of leucaena toxicity in ruminants in eastern Indonesia was conducted in the provinces of West and East Nusa Tenggara (NTB and NTT) in October 2011. Five villages were selected from each of the islands of Lombok, Sumbawa, Sumba and West Timor (Plate 6.1) where leucaena feeding was an established practice; up to 10 animals were sampled from each village (Table 6.1) including cattle (Bali bulls and cows: *Bos javanicus*; and Ongole bulls: *Bos indicus*), kacang goats (*Capra hircus*), and buffalo (*Bubalus bubalis*). The animals, belonging to local villagers, were tethered or housed in 'kandangs' (individual pens within a small shed) and fed fresh cut leucaena daily. The percentage of leucaena in diet was recorded and varied according to availability of forage at the end of the dry season. Animal breed, sex, age, and the approximate level of leucaena in diet were also recorded, as well as the number of days animals had been consuming leucaena prior to sampling (Table 6.1). This study was sanctioned under animal ethics #SAFS/144/11/ACIAR (Appendix 1).

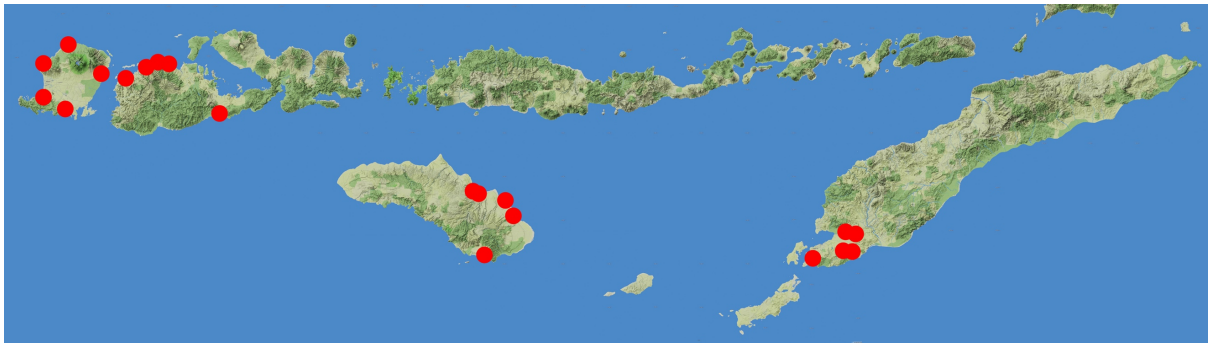

Plate 6.1: Locations of sample collection (●) in the islands of (from left to right): Lombok; Sumbawa; Sumba; and West Timor.

Table 6.1: General data on animals sampled, percentage of leucaena in diet and time consuming leucaena, animal age, and village location.

Island	Animal	Species	District	Village	GPS Coordinates		n	Approx. leucaena in diet (%)	Approx. time consuming leucaena (days)	Approx. age (months)	
										Mean	Range
Lombok	Cattle	*Bos javanicus*	North Lombok	Bayan	8º 13' 50.99" S	116º 19' 50.40" E	10	30	920	74	36–84
			West Lombok	Sekotong	8º 44' 17.40" S	116º 4' 34.01" E	9	30	500	36	36–36
	Goat	*Capra hircus*	Central Lombok	Rembitan	8º 50' 29.03" S	116º 17' 37.19" E	7	30	90	24	12–36
			East Lombok	Pringgabaya	8º 30' 51.60" S	116º 39' 22.40" E	9	100	4	11	11–13
			North Lombok	Bayan	8º 13' 50.99" S	116º 19' 50.40" E	8	30	600	36	36–36
			North Lombok	Pemenang	8º 24' 27.56" S	116º 3' 38.03" E	8	30	300	24	24–24
			West Lombok	Sekotong	8º 44' 17.40" S	116º 4' 34.01" E	9	30	217	19	12–36
Sumba	Buffalo	*Bubalus bubalis*	East Sumba	Kakaha	10º 14' 58.62" S	120º 22' 48.89" E	5	44	217	28	24–42
			East Sumba	Melolo	9º 52' 50.83" S	120º 39' 35.31" E	4	100	180	15	12–24
			East Sumba	Wanga	9º 46' 11.40" S	120º 36' 4.31" E	1	100	180	12	12–12
	Cattle	*Bos indicus*	East Sumba	Kakaha	10º 14' 58.62" S	120º 22' 48.89" E	3	20	365	24	24–24
			East Sumba	Wanga	9º 46' 11.40" S	120º 36' 4.31" E	2	100	730	24	24–24
	Goat	*Capra hircus*	Waingapu	Kamalaputi	9º 39' 5.86" S	120º 16' 4.67" E	7	50	28	12	12–12
			Waingapu	Kambaniru	9º 39' 51.59" S	120º 17' 38.58" E	6	90	639	21	12–24
Sumbawa	Cattle	*Bos javanicus*	Sumbawa	Jati Sari	8º 25' 10.20" S	117º 15' 56.09" E	10	92	39	24	18–48
			Sumbawa	Labangka I	8º 53' 49.58" S	117º 46' 8.88" E	8	93	202	17	12–30
			Sumbawa	Penyenger	8º 26' 7.05" S	117º 4' 59.56" E	9	74	473	25	13–36
			Sumbawa	Rhee	8º 24' 39.88" S	117º 13' 8.13" E	6	50	20	18	18–18
			West Sumbawa	Poto Tano	8º 32' 30.06" S	116º 52' 0.83" E	7	97	94	15	12–24
Timor	Cattle	*Bos javanicus*	Amarasi	Ponain	10º 12' 11.69" S	123º 51' 16.22" E	6	43	365	22	12–24
			Amarasi	Tesbatan II	10º 12' 44.81" S	123º 54' 17.65" E	6	80	365	21	12–24
			Fatuleu	Lili	10º 3' 49.73" S	123º 53' 2.65" E	10	70	365	30	24–48
			Fatuleu	Oelbeba	10º 4' 26.76" S	123º 58' 32.11" E	8	85	90	24	24–24
			West Kupang	Sumlili	10º 18' 1.53" S	123º 32' 53.09" E	4	79	60	24	24–24
	Goat	*Capra hircus*	West Kupang	Sumlili	10º 18' 1.53" S	123º 32' 53.09" E	6	100	180	26	12–36

6.2.2 Urine measurements

Urine was collected via voluntary urination following manual stimulation of the penis sheath in males or vulva in females. A soft brush was employed to encourage urination if necessary (Plate 6.2). A subsample of each urine collection was preserved by acidification with concentrated hydrochloric acid (HCl—32%) at a ratio of 1:19 and stored chilled. The preserved samples were exported to Australia and mimosine and DHP concentration measured using HPLC by the method described in Dalzell *et al.* (2012) with a corrected mobile phase of 25 mM ammonium dihydrogen phosphate. An additional peak in HPLC chromatograms was identified eluting prior to 2,3-DHP with a ≥98 similarity in absorption spectra in the wavelength range λ = 190–400 nm, which was suspected to be the conjugate form, indicative of incomplete acid-hydrolysis. Samples were also tested immediately on location using the colorimetric method of Graham *et al.* (2013).

Plate 6.2: Urine being collected from a Bali bull in Jati Sari, Sumbawa, using a plastic collecting cup and a soft brush to stimulate urination.

6.2.3 Rumen fluid collection

Rumen fluid samples were collected from up to 3 animals in each village via an orogastric tube and hand operated vacuum pump as described in Graham *et al.* (2013) (Plate 6.3). Rumen fluid was strained through 4 layers of muslin cloth into a collection container; a duplicate 3 mL sub-sample was withdrawn from the middle of the collection, and then

added to 7 mL of 100% ethanol and exported to Australia for DNA isolation. Remaining rumen fluid was used for the degradation study as described below.

Plate 6.3: Rumen fluid being collected from a buffalo in Melolo, Sumba, using a hand-operated orogastric pump.

6.2.4 Degradation study

Hungate tubes were prepared anaerobically as described in Hungate (1969) and modified in Bryant (1972). For fresh rumen fluid collection, tubes were prepared with 0.5 mL of 400 mg/L pure L-mimosine solution, as the substrate for degradation. A second batch of tubes was prepared with 0.5 mL of 400 mg/L pure L-mimosine solution added to 9 mL culture media containing modified medium 3,4A (Allison *et al.* 1992; McSweeney *et al.* 1993a). The resultant media contained the following: 0.5 mL of 400 mg/L pure L-mimosine solution; 30% (vol/vol) clarified rumen fluid; 3.8% (vol/vol) mineral solution 2 (Caldwell and Bryant 1966) with additional with 6 g/L K_2HPO_4; 3.8% (vol/vol) mineral Solution 3 consisting of 6 g/L KH_2PO_4, 6 g/L $(NH_4)_2SO_4$, 12 g/L NaCl, 2.5 g/L $MgSO_4 \cdot 7H_2O$, and 1.6 g/L $CaCl_2 \cdot 2H_2O$; 1 g/L resazurin; 1mL/L haemin; 1 g/L yeast extract, 1 g/L phytone peptone, 1 g/L peptone P; 2 g/L tryptone; 0.4 g/L cysteine hydrochloride; and supplemented with B-vitamins (McSweeney *et al.* 2005). The pH was adjusted to pH 6.8 with 2.5 M NaOH at ambient temperature prior to autoclaving at 121ºC (15 psi) for 15 minutes.

When available, a 9.5 mL sub-sample of strained rumen fluid was added to the prepared Hungate tubes containing 0.5 mL mimosine solution using a 21G needle, maintaining anaerobic environment with a 25G needle allowing backflow of gas. Where rumen fluid was limited or unattainable, as was the case on the island of Sumbawa and Timor, either 0.5 mL of rumen fluid or 0.5 mL of strained fresh faeces liquor was used to inoculate tubes prepared with 9.0 mL of culture medium and 0.5 mL of 400 mg/L pure L-mimosine solution.

All tubes were incubated, initially in a portable esky maintained at 39ºC (±2ºC) through a thermostat-controlled heating element and fan while collections took place. Tubes were later transferred into an incubator, maintained at 39ºC on a rocker. Sub-samples were taken of 21 and 28 days after inoculation to measure DHP degradation. Hungate tubes were inverted and a 2 mL aliquot was withdrawn using a 21G needle and dispensed into 2 mL Eppendorf tubes. These were spun at 1300 × g for 15 mins. The supernatant was withdrawn and frozen at <−20ºC prior to analysis by HPLC.

6.2.5 DNA isolation and detection of Synergistes jonesii

Rumen fluid samples, exported to Australia preserved in ethanol, were used for DNA isolation. DNA extraction was performed with detection of *S. jonesii* by primary and nested PCR, according to the method described in Chapter 5. Sequencing of positive nested PCR products was aligned with the 16S rDNA sequence of the *S. jonesii* type strain (ATCC 49833 (78-1)). Samples identical to the type strain (without any single nucleotide polymorphisms — SNPs) were noted.

6.2.6 Statistical analyses

Data were analysed using Microsoft Excel computer software (©2016, Microsoft, Redmond, WA, USA) and Minitab® statistical software 16 (©2010, Minitab Inc., State College, PA, USA).

One-way ANOVA tests were undertaken for urinary 3,4-DHP and 2,3-DHP levels in comparison with: the presence of *S. jonesii*; presence of the ATCC type strain; island; and species of ruminant. The presence of *S. jonesii* and the presence of the ATCC type strain were analysed with an ANOVA using a GLM including island, species, and type of sample collected. Significant terms were further analysed using a one-way ANOVA with pairwise comparisons made using Fishers least significant difference (LSD) method.

Regression analysis was undertaken for urinary 3,4-DHP, urinary 2,3-DHP, presence of *S. jonesii*, and presence of the ATCC type strain, with respect to the time animals had been consuming leucaena, and the amount of leucaena in diet. A GLM ANOVA model was used for repeated measures analysis for results of the degradation study, with respect to species, island and sampling time.

Descriptive statistics were used to summarise mean values with standard errors listed. A probability level of $P < 0.05$ was considered statistically significant, while a *P*-value between 0.05–0.10 was considered a moderate trend.

6.3 Results

6.3.1 Diet composition

The amount of leucaena in diet varied among villages, with farmers offering between 20–100% leucaena (Table 6.1), with most animals fed >30% leucaena in diet. Percentage of leucaena in diet was dependant on individual farmer management, forage availability, and their perceived value of leucaena as a forage. In general, levels of leucaena in diet were lower than usual, as the amount of leucaena available was limited due to the dry season.

6.3.2 Urinary DHP excretion

The mean, and range, of urinary DHP excretions are presented in Table 6.2 by island and by species. Mimosine was not detected in any sample. The dominant isomer of DHP excreted was 2,3-DHP comprising a mean of 77% of all DHP excreted, consistent across all species, with the exception of *Bos indicus* cattle from Sumba, where both percentage of leucaena in diet and DHP excretions were low. The range in 2,3-DHP excretions was also 10 times greater than that of 3,4-DHP. One-way ANOVA was conducted to establish possible linkages. There were no significant differences in level of 3,4-DHP excretion due to the detection of *S. jonesii* (P = 0.45) or to the presence of the ATCC type strain (P = 0.43) in rumen fluid. Likewise, there were no significant differences in level of 2,3-DHP excretion due to detection of *S. jonesii* (P = 0.13) or to the presence of the ATCC type strain (P = 0.15) in rumen fluid. There were also no significant differences in 3,4-DHP excretion due to island (P = 0.33) or to species (P = 0.39). For 2,3-DHP excretion however, while there was no significant difference among species (P = 0.18), there was a significant effect due to island (P < 0.01) with Sumba having the lowest concentrations (Table 6.3).

One-way ANOVA regression analyses indicated no trend in 3,4-DHP excretion with time on leucaena (months) (P = 0.79) or with amount of leucaena in diet (%) (P = 0.13). There was also no trend in 2,3-DHP concentrations with time on leucaena (months) (P = 0.97), but there was a significant (P = 0.00), though weak relationship (R^2 = 0.08), with amount of leucaena in diet (%) (2,3-DHP = 918 × leucaena in diet + 36).

Table 6.2: Mean (±s.e.) and range of urinary DHP excretions from animals as determined by HPLC, and mean percentage of 2,3-DHP that remained conjugated after hydrolysis.

Island	Animal	Species	District	Village		3,4-DHP (mg/L)		2,3-DHP (mg/L)			Total DHP (mg/L)	
					n	Mean±s.e.	Range	Mean±s.e.	Range	Mean % remaining conjugated	Mean±s.e.	Range
Lombok	Cattle	*Bos javanicus*	North Lombok	Bayan	8	4±2	0–14	10±6	0–34	4	14±8	0–52
			West Lombok	Sekotong	9	116±30	0–226	622±192	0–1506	25	738±216	0–1680
	Goat	*Capra hircus*	Central Lombok	Rembitan	7	262±86	34–806	1812±490	368–4778	13	2074±554	402–5236
			East Lombok	Pringgabaya	9	10±4	0–32	0±0	0–0	47	12±4	0–32
			North Lombok	Bayan	10	258±82	0–794	1680±512	122–4480	20	1938±588	122–5274
			North Lombok	Pemenang	8	16±10	0–66	342±226	0–1416	0	358±236	0–1484
			West Lombok	Sekotong	9	126±100	0–908	1090±716	0–6304	24	1216±806	0–7214
Sumba	Buffalo	*Bubalus bubalis*	East Sumba	Kakaha	2	68±24	44–90	270±148	122–416	36	336±124	212–460
			East Sumba	Melolo	4	104±14	74–136	268±72	186–482	40	372±84	264–618
			East Sumba	Wanga	1	32±	32–32	92±	92–92	45	124±	124–124
	Cattle	*Bos indicus*	East Sumba	Kakaha	2	10±10	0–20	0±0	0–0	0	18±4	14–20
	Goat	*Capra hircus*	Waingapu	Kamalaputi	6	4±4	0–20	22±12	0–72	23	24±12	0–72
			Waingapu	Kambaniru	5	28±10	0–62	72±58	0–298	21	100±68	0–360
Sumbawa	Cattle	*Bos javanicus*	Sumbawa	Jati Sari	9	144±82	0–450	2284±1058	0–5920	41	2430±1126	0–6370
			Sumbawa	Labangka I	8	400±52	204–646	2238±570	652–5220	35	2650±594	888–5742
			Sumbawa	Penyenger	9	190±54	0–418	2798±1254	0–10144	32	2998±1302	16–10558
			Sumbawa	Rhee	6	348±138	0–1092	2536±1184	40–7670	23	2886±1316	40–8772
			West Sumbawa	Poto Tano	7	220±36	108–464	1384±406	294–3360	33	1606±438	402–3796
Timor	Cattle	*Bos javanicus*	Amarasi	Ponain	6	28±6	18–38	72±30	16–156	13	98±36	34–192
			Amarasi	Tesbatan II	5	300±62	112–544	1774±412	374–3034	35	2076±464	486–3422
			Fatuleu	Lili	4	92±70	0–432	700±450	0–2438	27	792±482	0–2438
			Fatuleu	Oelbeba	6	152±52	54–256	592±270	130–1288	40	746±320	184–1544
			West Kupang	Sumlili	4	94±40	0–202	772±404	0–1952	38	866±432	0–2070
	Goat	*Capra hircus*	West Kupang	Sumlili	4	620±146	358–1040	4994±786	3224–6962	44	5614±928	3582–8002

Table 6.3: Mean 2,3-DHP excretion levels by island.

Means with different subscripts are significantly different at $P < 0.05$

Island	Mean 2,3-DHP excretion (mg/L)
Lombok	852_{bc}
Sumba	94_{c}
Sumbawa	2230_{a}
Timor	1426_{ab}

6.3.3 Degradation study

The mean mimosine and 3,4-DHP levels for each animal species within islands at 0, 21 and 28 days after inoculation are presented in Table 6.4. The initial concentration of mimosine (~17 mg/L) was consistent among tubes. No mimosine was present from 21 days post-inoculation regardless of method of inoculation. Also notable was the lack of presence of 2,3-DHP, regardless of source of inoculum or time sampled. With the exception of a single bull from Timor, all samples inoculated with 9.5 mL of fresh rumen fluid resulted in complete degradation after 21 days (Table 6.4a) (Figure 6.1). There was no significant difference between day 21 and 28, nor between species or islands.

In contrast, when 9.5 mL of culture media with mimosine was inoculated with 0.5 mL of rumen fluid or strained faeces, not all 3,4-DHP was degraded (Table 6.4b) (Figure 6.2). The isomer 3,4-DHP was detected at 21 and 28 days post-inoculation in culture media tubes; but there was no significant difference in degradation between these sampling times. This indicated that in culture media, faeces microbes possessed the same efficacy in degrading 3,4-DHP as rumen fluid. However, since mean degradation was only ~35% at 28 days, more time may be required to achieve complete degradation with only 0.5 mL of inoculum. Analysis of variance revealed no significant differences among islands (Figure 6.2) and no significant difference in degradation capability. However, the percentage of 3,4-DHP degraded was significantly greater when inoculated with rumen fluid from Sumba buffalo, compared to rumen fluid from Sumba goats or from cattle faeces samples from Sumbawa and Timor ($P < 0.001$) (Table 6.4b).

Table 6.4: Mean mimosine and 3,4-DHP concentrations (mg/L) of sub-samples from Hungate tubes from the degradation study at 0, 21, and 28 days after inoculation with either: (a) 9.5 mL of rumen fluid, or (b) 0.5 mL of rumen fluid or faeces liquor into culture media.

(a) Inoculation of 9.5 mL of rumen fluid into 0.5 mL 400 mg/L mimosine

Means within columns with differing subscripts denote significant differences at the 5% significance level

Sample type	Island	Animal	Species	n	Mean mimosine (mg/L) days after inoculation			Mean 3,4-DHP (mg/L) days after inoculation			Mean±s.e final degradation (%)
					0	21	28	0	21	28	
Rumen fluid	Lombok	Cattle	*Bos javanicus*	5	17	0	0	0	0_a	0_a	$100\pm0_a$
		Goat	*Capra hircus*	11	17	0	0	0	0_a	0_a	$100\pm0_a$
	Sumba	Buffalo	*Bubalus bubalis*	5	17	0	0	0	0_a	0_a	$100\pm0_a$
		Cattle	*Bos indicus*	2	17	0	0	0	0_a	0_a	$100\pm0_a$
		Goat	*Capra hircus*	3	17	0	0	0	0_a	0_a	$100\pm0_a$
	Timor	Cattle	*Bos javanicus*	3	17	0	0	0	3_a	3_a	$83\pm17_a$
		Goat	*Capra hircus*	2	17	0	0	0	0_a	0_a	$100\pm0_a$

(b) Inoculation of 0.5 mL rumen fluid or faeces liquor into 9.0 mL culture media and 0.5 mL 400 mg/L mimosine

Means within columns with differing subscripts denote significant differences at the 5% significance level

Sample type	Island	Animal	Species	n	Mean mimosine (mg/L) days after inoculation			Mean 3,4-DHP (mg/L) days after inoculation			Mean±s.e Final degradation (%)
					0	21	28	0	21	28	
Rumen fluid	Sumba	Buffalo	*Bubalus bubalis*	3	17	0	0	0	8_a	8_a	$57\pm20_a$
		Cattle	*Bos indicus*	1	17	0	0	0	11_{ab}	11_{ab}	39_{ab}
		Goat	*Capra hircus*	3	17	0	0	0	11_b	14_b	$24\pm2_b$
	Timor	Cattle	*Bos javanicus*	7	17	0	0	0	10_{ab}	12_{ab}	$33\pm4_{ab}$
		Goat	*Capra hircus*	1	17	0	0	0	13_{ab}	12_{ab}	31_{ab}
Faeces	Sumbawa	Cattle	*Bos javanicus*	15	17	0	0	0	11_b	12_b	$34\pm1_b$
	Timor	Cattle	*Bos javanicus*	7	17	0	0	0	11_b	12_b	$29\pm3_b$

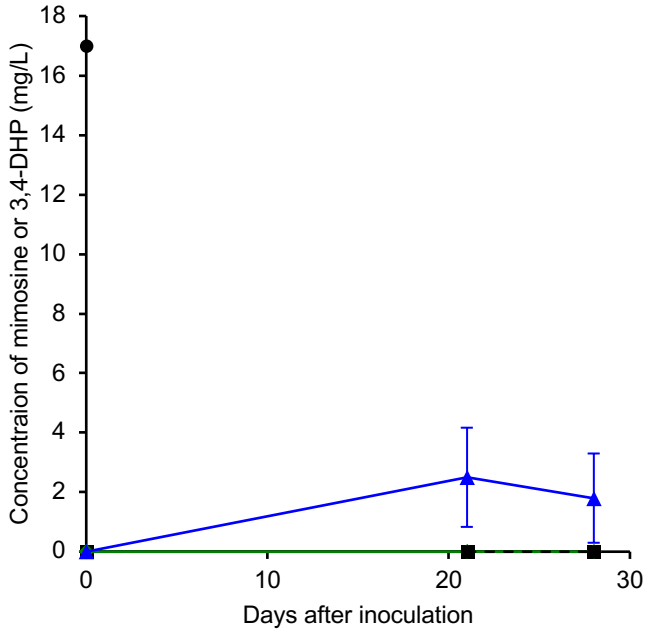

Figure 6.1: Degradation of mimosine [all islands (●)] and 3,4-DHP (solid lines) during the incubation period for tubes inoculated with 9.5 mL of fresh rumen fluid for: Lombok (■); Sumba (◆); Timor (▲).

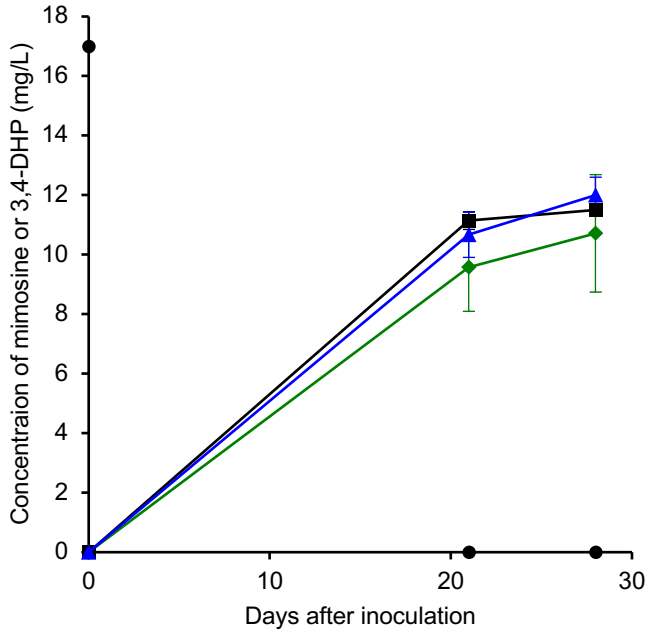

Figure 6.2: Degradation of mimosine [all islands (●)] and 3,4-DHP (solid lines) during the incubation period for tubes containing culture medium, inoculated with 0.5 mL of rumen fluid for: Sumba (◆); Sumbawa (■); Timor (▲).

6.3.4 Detection of S. jonesii *and ATCC type strain*

The detection rates of *S. jonesii* and presence of the ATCC type strain, collected from rumen fluid and from faeces are presented in Table 6.5a and Table 6.5b. Of the 66 animals sampled, 45% were positive for *S. jonesii*; 40% of all positive samples were also positive for the ATCC type strain after nested PCR sequencing (18% of total animals sampled). A one-way analysis of variance, indicated detection of *S. jonesii* was significantly higher ($P < 0.001$) in rumen fluid samples (66%) compared to faeces samples (5%); only 1 of the 23 faeces samples was positive for *S. jonesii*. The lack of detection of *S. jonesii* in samples from Sumbawa island was due to the collection of faeces only. There was also a significant difference ($P = 0.04$) in presence of the ATCC type strain in rumen fluid (25%), compared to faeces (5%), with only 1 of the 23 faeces samples positive for the ATCC type strain.

An analysis of variance (GLM) showed that there was no significant difference in detection rate for *S. jonesii* due to island ($P = 0.88$); however, there was a significant difference between species ($P < 0.001$) (Table 6.6); detection rate in buffalo and goats was greater than in Bali cattle. There was no significant difference in detection of the ATCC type strain due to island ($P = 0.55$); however, there was a significant ($P < 0.001$) difference in detection rate due to animal species, with greater detection in buffalo, compared to Bali cattle and goats (Table 6.7).

There were no significant trends when the detection rate of *S. jonesii* was regressed against percentage of leucaena in diet or time consuming leucaena; $P = 0.23$ and $P = 0.06$, respectively. Similarly, the detection rate of the ATCC type strain was also not related to percentage of leucaena in diet or time consuming leucaena ($P = 0.98$ and $P = 0.84$, respectively).

Table 6.5: Number of (a) rumen fluid, and (b) faeces samples, tested positive for *Synergistes jonesii* and positive for the ATCC type strain.

(a) Rumen fluid

Island	Animal	Species	District	Village	*n* (rumen fluid)	Animals positive for *S. jonesii*	Animals positive for ATCC type strain	*S. jonesii* detection rate (%)	ATCC type stain detection rate (%)
Lombok	Cattle	*Bos javanicus*	North Lombok	Bayan	3			0	0
			West Lombok	Sekotong	2			0	0
	Goats	*Capra hircus*	Central Lombok	Rembitan	3	3	1	100	33
			East Lombok	Pringgabaya	3	3		100	0
			North Lombok	Pemenang	3	3		100	0
			West Lombok	Sekotong	2	2		100	0
Sumba	Buffalo	*Bubalus bubalis*	East Sumba	Kakaha	3	1	1	33	33
			East Sumba	Melolo	3	3	3	100	100
			East Sumba	Wanga	1	1	1	100	100
	Cattle	*Bos indicus*	East Sumba	Kakaha	1	1	1	100	100
			East Sumba	Wanga	2	1		50	0
	Goats	*Capra hircus*	East Sumba	Kamalaputi	3	3		100	0
			East Sumba	Kambaniru	3	2		67	0
Timor	Cattle	*Bos javanicus*	Amarasi	Ponain	3	2	2	67	67
			Amarasi	Tesbatan II	3	2	2	67	67
			Fatuleu	Lili	3			0	0
	Goats	*Capra hircus*	West Kupang	Sumlili	3	2		67	0

(b) Faeces

Island	Animal	Species	District	Village	*n* (faeces)	Animals positive for *S. jonesii*	Animals positive for ATCC type strain	*S. jonesii* detection rate (%)	ATCC type stain detection rate (%)
Sumbawa	Cattle	*Bos javanicus*	Sumbawa	Jati Sari	3			0	0
			Sumbawa	Labangka I	3			0	0
			Sumbawa	Penyenger	3			0	0
			Sumbawa	Rhee	3			0	0
			West Sumbawa	Poto Tano	3			0	0
Timor	Cattle	*Bos javanicus*	Fatuleu	Lili	2	1	1	50	50
			Fatuleu	Oelbeba	5			0	0

Table 6.6: Mean (±s.e.) detection rate of *S. jonesii* from rumen fluid collected from each species across all islands.

Means with differing subscripts denote significant differences at the 5% significance level

Species	Mean±s.e. *S. jonesii* detection rate (%)
Bubalus bubalis	$71 \pm 13_{a}$
Bos indicus	$67 \pm 33_{ab}$
Bos javanicus	$29 \pm 13_{b}$
Capra hircus	$88 \pm 6_{a}$

Table 6.7: Mean (±s.e.) detection rate of the ATCC type strain from rumen fluid collected from each species across all islands.

Means with differing subscripts denote significant differences at the 5% significance level

Species	Mean±s.e. ATCC type strain detection rate (%)
Bubalus bubalis	$71 \pm 13_{a}$
Bos indicus	$33 \pm 33_{ab}$
Bos javanicus	$29 \pm 13_{b}$
Capra hircus	$6 \pm 4_{b}$

6.4 Discussion

The major finding of this study was that in eastern Indonesia, previously considered protected from leucaena toxicity by *S. jonesii* (Jones 1994), animals regularly consuming high leucaena diets were not completely degrading DHP. Although *S. jonesii*, the most widely accepted DHP-degrading rumen microbe, was detected in the majority of rumen fluid samples (Padmanabha *et al.* 2014), it was however, present in low populations often at or below the limit of detection using PCR techniques (Graham *et al.* 2013). The absence of mimosine in urine, in association with high levels of DHP indicate that ruminants possesses sufficient microbial capacity to degrade mimosine, however insufficient rumen microbes to fully degrade the resultant DHP. Despite animals having DHP levels considered high enough to cause severe toxicosis, no animals were exhibiting signs of clinical or subclinical toxicity. As there was evidence of conjugation (Table 6.2), it is possible that this may have contributed to the detoxification of DHP and lack of symptoms. These findings are now discussed.

6.4.1 High levels of DHP in urine

In ~75% of all locations sampled, animals were classified as excreting levels of DHP exceeding the threshold for subclinical toxicity of 100 mg/L (Dalzell *et al.* 2012) with some individual animals excreting very high levels, exceeding 10000 mg/L (Table 6.2). This result strongly contrasts the previously considered protection status for ruminants on high leucaena diets in eastern Indonesia, where *S. jonesii* was considered endemic (Jones 1994) and capable of degrading 100% of DHP (Jones and Lowry 1984). However, the findings of this study are in accord with other more recent studies in Indonesia (Halliday *et al.* 2013; Halliday *et al.* 2014b)(Chapter 4), Thailand (also originally considered protected) (Phaikaew *et al.* 2012) and Mexico (Contreras-Hernández *et al.* 2013; Ruz-Ruiz *et al.* 2013).

While the incidence of high levels of DHP should indicate potential serious health issues (Jones and Winter 1982; Jones and Bray 1983; Quirk *et al.* 1988), no clinical signs of toxicity were observed with animals in excellent condition. Although liveweight gain (LWG) was not recorded, farmers reported satisfaction with the growth performance of the animals, and there was no effect on breeding performance. Farmers did however note that there were usually initial signs of mimosine and DHP toxicity, but these resolved within 3–4 weeks of adaptation to leucaena consumption. This adaptation period was reported to be common and normal, especially in cut-and-carry systems in Sumbawa, where 100%

leucaena is often fed over a 4–6 month period (Panjaitan *et al.* 2013). The variability exhibited in urinary DHP excretions within and among locations was also normal, as time after consuming leucaena, hydration and fullness of bladder, and individual animal metabolic rates have resulted in large differences in urine concentration of DHP due to sampling time (Guyton and Hall 2000; Giles *et al.* 2013).

Also contradicting the original work on leucaena toxicity, was the finding of high levels of 2,3-DHP in urine in this study. The presence of 2,3-DHP was originally considered novel (Ford *et al.* 1984) and transitory (Jones *et al.* 1985a); however, it is clear that it is a regular end-product of ruminal metabolism of mimosine. Occurrence of 2,3-DHP has been recorded in many countries. A meta-analysis conducted by Halliday *et al.* (2014b) showed this to be the case in Australia (Dalzell *et al.* 2012; Graham *et al.* 2013; Halliday *et al.* 2014a), India (Ghosh *et al.* 2007), Thailand (Phaikaew *et al.* 2012) and Indonesia (Halliday *et al.* 2014c). Thus, it is concluded that 2,3-DHP is the predominant isomer excreted in urine, and it is present at very high concentrations; enough to potentially cause chronic toxicity.

6.4.2 S. jonesii *is present in low numbers*

The results of this study also support the more recent discoveries that there is ubiquitous rather than isolated distribution of *S. jonesii* (Padmanabha *et al.* 2014)(Chapter 5). *S. jonesii* was detected, at low population levels, using rDNA PCR in the majority of rumen fluid samples collected (~66% of rumen samples), in all islands and species, despite high urinary DHP levels detected in most animals.

Although *S. jonesii* was not detected in 100% of rumen fluid samples, all Hungate tubes inoculated with 9.5 mL of fresh rumen fluid were capable of complete degradation of small amounts of mimosine within 21 days; this was positive evidence for the presence of DHP-degrading microbes. Jones *et al.* (2009) conducted a similar experiment and found 100% degradation of DHP in fresh ground leucaena within ~4 days, using a greater volume of rumen fluid (20×) and larger mimosine concentrations (1000×). As the theoretical limit of detection for *S. jonesii* is between 10^4–10^5 cells/mL in rumen digesta (Graham *et al.* 2013), it was therefore apparent that *S. jonesii* was present in all animals, however at populations at or below the limit of detection using current methodologies.

Thus, although the indigenous nature of *S. jonesii* would imply no inoculation would be required to transfer the microbe, the lack of complete degradation *in vivo* and the ubiquitously low populations, evidenced in this study, suggest *S. jonesii* would not be

capable of fully protecting ruminants on high leucaena diets. The results of the inoculation study in Chapter 4, where DHP levels remained high in recipient animals 10-days post-inoculation with fresh rumen fluid, support this assertion.

Detection of *S. jonesii* was even lower in faeces samples (~5%) and the degradation of mimosine *in vitro* with culture media inoculated with 0.5 mL of rumen fluid or 0.5 mL of faeces liquor was not capable of complete degradation within the 28-day monitoring period, achieving only approximately 32–36% degradation. This was likely due to the methodological limitations of the culture media, receiving ~0.3% of the volume, and hence a lower initial population of microbes, as those inoculated with rumen fluid only. It was thus clear from this study that *S. jonesii* does survive in the faeces at very low levels and may require extended periods to colonise and build the capacity to degrade DHP. Transfer of *S. jonesii* encapsulated in the faeces has been suggested as a possible vector of migration of 'the bug' throughout cattle herds (Pratchett *et al.* 1991).

This study confirms the fundamental ability of *S. jonesii* to degrade DHP; the results of the degradation study using 9.5 mL of rumen fluid strongly support other work on the DHP-degrading ability of *S. jonesii* (Allison *et al.* 1990; Klieve *et al.* 2002). However, there is considerable evidence that *S. jonesii* does not have the capability to completely degrade all DHP when ruminants are daily consuming high leucaena diets. The absence of any 2,3-DHP *in vitro*, despite it being the predominant isomer excreted *in vivo*, further suggests that the static, artificial nature of *in vitro* studies cannot be directly extrapolated to the continuous flow of high levels of mimosine/DHP entering the rumen. A notable example in this study includes rumen fluid collected from individual goats in Sumlili, West Timor, positive for *S. jonesii*, with SNPs detected, that were capable of 100% degradation *in vitro*; however, *in vivo* urinary DHP excretions were >7600 mg/L.

What is not clear however, is the reason for the high levels of 2,3-DHP in urine. The presence of 2,3-DHP infers the presence of a microorganism capable of the isomerisation of 3,4-DHP. However, by inference from PCR assay, the populations of *S. jonesii* were insufficient to completely degrade DHP in animals consuming high leucaena diets. To explain this paradox, three new hypotheses are suggested: (1) that other microbes, such as those described in Hammond *et al.* (1989), Domínguez-Bello and Stewart (1991), Tan *et al.* (1994), Chhabra *et al.* (1998), Aung *et al.* (2011) and Derakhshani *et al.* (2016) are present contributing to the isomerisation of 3,4-DHP; (2) that the many variants of *S. jonesii* identified have differential functional capacity to degrade individual isomers, and these molecular variants cannot metabolise 2,3-DHP (Halliday *et al.* 2014b); or, (3) that

the conversion of 3,4-DHP to 2,3-DHP is not an essential intermediary in the complete degradation of DHP. It is inconclusive as to what mechanisms are operating and further investigation is warranted.

6.4.3 The animals on high leucaena diets were healthy

Despite high levels of DHP in urine, there were no apparent deleterious effects on animals consuming high leucaena diets. Production levels were not only unaffected but greatly increased when leucaena was fed. There were no indications of toxicity apart from the initial alopecia, when animals were first introduced to leucaena, due to mimosine toxicity. This clinical sign disappeared in 2–4 weeks. Other studies have reported high weight gains achieved on high leucaena diets, even though high levels of DHP were being excreted (Panjaitan *et al.* 2014). Also, the lack of reproductive impairment, normally sensitive to toxicity, suggests that animals were not experiencing DHP toxicosis. It was clear the presence of *S. jonesii* was insufficient to elicit full protection, yet animals were not adversely affected by DHP. This provides evidence for the hypothesis that an alternate method of protection is involved, either in concert with or independent of *S. jonesii*.

It is postulated that hepatic conjugation is occurring, a natural mechanism for detoxifying hydroxyl compounds (Smith 1971) such as DHP. The process of conjugation has a two-fold effect on detoxification. Firstly, it increases the polarity of the molecule, and therefore water-solubility, increasing urinary clearance of the toxin. Secondly, it is hypothesised that conjugation reduces the ion-binding affinity of the hydroxyl group/s, reducing the acute toxicity of the molecule (Tsai and Ling 1971; Ghosh and Samiran 2007). The common conjugation mechanism in mammals is that of glucuronidation, which comprises the attachment of a glucose-based carboxylic acid: glucuronic acid.

The methodology employed in this study was not able to measure initial levels of conjugated DHP at time of excretion, as the acidification of samples initiated hydrolysis of the conjugated DHP. Nevertheless, despite sample preparation involving an additional acidification step (Graham *et al.* 2014), after acid hydrolysis, ~27% (range 0–47%) of total 2,3-DHP remained in the conjugated form (Table 6.2). The fact that animals were not suffering from clinical or subclinical toxicity, despite in some cases, excreting exceedingly high levels of DHP, suggested that much of the DHP may have been in the non/less toxic conjugated form at time of excretion, and therefore was not contributing towards the animals' toxicity status.

The amelioration of toxins via conjugation is not a novel phenomenon and occurs in all mammals for a variety of xenobiotics (Smith 1971; Christie *et al.* 1979; Elliott *et al.* 1985). First postulated as a coping mechanism for animals consuming leucaena (Hegarty *et al.* 1979), there has been little focus on this mechanism since. However, if the hypothesis put forward is correct—that conjugation of DHP is a major pathway in DHP detoxification—it represents a major paradigm shift from current leucaena management practices and is good news for leucaena feeding operations worldwide.

6.4.4 Limitations

A limitation of measurements of DHP concentration in urine is the inherent diurnal dilution factor involved with urine excretions and the potential to lead to the risk of false toxicity assessment. The continuous monitoring study of Giles *et al.* (2013) recorded a 50 fold difference (138–6932 mg/L) in minimum and maximum DHP concentrations of 34 individual samplings over a 24-hour period (total urine volume 16.9 L) in a steer consuming 100% leucaena. Thus, a single sample in isolation could lead to false assessment of the actual DHP load animals were experiencing.

The other limitation of this study, associated with the single collection obtained from individual animals was the possibility that animals were in a period of transition where animals were adapting to leucaena (Jones *et al.* 2009). This was unlikely as samples were collected at differing times of the day for different animals, and many animals had been on high leucaena diets for extended periods. This study sought to overcome these limitations by assessing mean DHP levels of a population of 10 animals per species, per location. Nevertheless, it should be noted that the unusually low DHP levels recorded in Sumba, although coupled with high detection rate of *S. jonesii*, could possibly be an effect of smaller sample sizes on that island.

This study has highlighted contradictions to the original theory that ruminants in only specific countries were protected by *S. jonesii* (Jones 1981). As the original survey work relied on urinary DHP tests from few animals where there was likely incomplete and in some cases minimal hydrolysis of the bound DHP (Jones 1994), the validity of urinary free-DHP testing in isolation is questioned as it does not profile other mechanisms that the animal may be employing to adapt to DHP in diet. Furthermore, the classification of an entire country based on isolated testing does not reflect the myriad feeding systems utilised within these countries.

6.5 Conclusions

In conclusion, this study postulates a new paradigm in toxicity management for ruminants consuming high leucaena diets. It is apparent that inoculation with *S. jonesii* is not a prerequisite for protection against toxicity for two reasons: (1) it is already an indigenous microbe in all ruminants; and (2) it is not capable of completely detoxifying DHP in animals on high leucaena diets, due to its presence at low populations in the rumen. In spite of the poor efficacy of *S. jonesii*, and the resultant high levels of DHP excreted, animal health and production in these herds were not adversely affected. The hypothesis put forward is that animals were coping with undegraded DHP via the mechanism of hepatic conjugation with glucuronic acid. Although the methodology of this study was not designed to quantify levels of conjugation, there was evidence that conjugation was occurring.

The significance of this hypothesis, if confirmed, cannot be understated as leucaena-based feeding systems have the capacity to double animal production, while halving the labour requirements of forage collection in developing tropical countries. Ruminal inoculation with *S. jonesii* is not a feasible option in developing countries (Chapter 4), and to safely avoid the need for inoculation would be an important economic outcome.

Further studies are recommended to ascertain the significance of conjugation as a detoxification mechanism. As the isomer 2,3-DHP is of greatest abundance, improved understanding as to its mode of toxicity, and relative toxicity to 3,4-DHP is warranted. Future work should determine the levels of conjugation of excreted DHP and the effect of conjugation of DHP on the ion-binding affinity. As the binding and depletion of essential metals is a primary mode of toxicity, mineral supplementation may also assist to overcome toxicity.

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Chapter 7 Liveweight gain, dry matter intake and urinary hydroxypyridone (DHP) levels of *Capra hircus* goats in response to diets of *Gliricidia sepium* infused with DHP and *Leucaena leucocephala*, with and without iron(II) sulphate mineral supplementation.

7.1 Introduction

As many smallholder ruminant feeding systems in eastern Indonesia rely on *Leucaena leucocephala* (leucaena) for vital protein nutrition, especially into the extended dry seasons, the amelioration of toxicity is of great importance to maximise the positive benefits of the forage. Toxicity in leucaena is due to the effects of mimosine and its breakdown products: 3-hydroxy-4(1*H*)-pyridone (Hegarty *et al.* 1976) and its structural isomer 3-hydroxy-2(1*H*)-pyridone (Ford *et al.* 1984; Jones and Megarrity 1986; Allison *et al.* 1992; McSweeney *et al.* 2002), commonly referred to as 3,4-DHP and 2,3-DHP, respectively. Early toxicity research focussed on the 3,4- isomer (Christie *et al.* 1979; Lee *et al.* 1980), and although 2,3-DHP was perceived to be transitory (Jones and Megarrity 1983; Jones *et al.* 1985a), it was considered similarly toxic (Lowry *et al.* 1985). The toxic nature of DHP can be attributed to its structure as a strong ligand, readily chelating with transition metal ions, which are mobilised and excreted in the urine (Tsai and Ling 1971; Stunzi *et al.* 1980; Hider 1995). As such, toxicity, when manifest, presents as the side effects associated with deficiencies of essential elements, including Zn, Cu and Fe (Mills 1978; Puchała *et al.* 1996; Paul 2000; Perry *et al.* 2005); this can contribute to the development of goitre (Nishi *et al.* 1980; Lukaski *et al.* 1992; Smith *et al.* 1992).

To counteract the deficiencies, mineral supplementation with iron(II) (Ross and Springhall 1963), or in combination with other bivalent metals including copper(II), zinc(II), and magnesium(II) (Jones *et al.* 1978; Puchała *et al.* 1995) was an early strategy for amelioration of leucaena toxicity. This was shown to increase dry matter intake (DMI) and liveweight gain (LWG), and to reduce clinical signs of DHP toxicosis (Tsai and Ling 1974; Jones *et al.* 1978; Puchała *et al.* 1995).

Despite the reported success, research into mineral supplementation waned after the discovery of the DHP-degrading rumen microbe *Synergistes jonesii* (Jones and Megarrity 1983; Jones and Lowry 1984). However, it has been demonstrated in earlier chapters (Chapters 4 & 5) that the efficacy of *S. jonesii* is limited in ruminants on high leucaena diets and, in light of the possible alternate metabolic detoxification mechanisms, a new paradigm has been proposed (Chapter 6). It is now postulated that detoxification of excess

DHP also occurs by either intraruminal chelation with available metal ions and/or hepatic conjugation post-ingestion.

A consistent outcome of recent research is the accumulation of high levels of the isomer 2,3-DHP (Ghosh *et al.* 2007; Dalzell *et al.* 2012; Phaikaew *et al.* 2012; Graham *et al.* 2013), without apparent adverse effects on animal health or performance (Chapters 4, 5 &6). Accordingly, controlled feeding trials were conducted to investigate the toxicity response to the now predominant isomer 2,3-DHP in goats and the impact of Fe(II) mineral supplementation on reducing its toxicity.

Two experiments were undertaken with goats as they are commonly fed leucaena in smallholder systems in eastern Indonesia and they represent a model small ruminant. In experiment 1, goats were infused with 2,3-DHP, while in experiment 2, goats received a single element mineral supplementation of Fe(II) sulphate, chosen to promote chelation of DHP as a prophylactic measure. Dosage was designed to maintain a net positive balance of available cations, rather than to overcome accumulated element deficiencies. The justification for the work was that, if mineral supplementation proved effective, it would represent a huge advance in the control of toxicity worldwide.

7.2 Materials and methods.

Experiment 1 primarily investigated the impact of 2,3-DHP infusion, with a short-term supplementary study added to investigate the effect of mineral supplementation on DHP excretion level and conjugation in goats infused with 2,3-DHP. Experiment 2 investigated the impact of longer-term mineral supplementation on goat performance. The experiments were conducted at a research farm in Sumlili village, West Kupang district, West Timor island, East Nusa Tenggara (NTT) province, Indonesia (10º 18' 2.10" S, 123º 32' 53.61" E).

It was hypothesised that: (a) directly infused free 2,3-DHP would have greater toxicity than 2,3-DHP originating from diets comprising a high percentage of leucaena; and (b) the chemical bonding of supplemented Fe(II) with DHP intraruminally would assist in preventing chelation of essential elements and subsequent metal deficiencies. In addition, the bonded DHP would be more efficiently excreted due to an increase in water-solubility (Galanello 2007; Sooriyaarachchi and Gailer 2010), thus minimising time spent in the body and reducing the overall toxic effect.

A control legume diet of gliricidia was provided at similar levels to standardise the legume intake of animals supplemented with 2,3-DHP and/or minerals. Gliricidia has a similar crude protein content to leucaena (Dahlanuddin 2001) and is widely fed to ruminants in eastern Indonesia (Shelton *et al.* 2005). It is a regular component of diets for goats in West Timor, where it is planted extensively as a living fence (Dahlanuddin *et al.* 2014).

7.2.1 Treatments and animal management

Experiment 1

Experiment 1 was a 7-week study and consisted of 4 treatments with 4 replications (Table 7.1) using 16 weaner kacang goats (*Capra hircus*) (average starting weight 12.7 ± 2.6 kg (±s.d.)) commencing in May 2012. Treatments are shown in Table 7.1 and described as follows:

- A control treatment of 50% *Gliricidia sepium* (gliricidia) and 50% local grasses, primarily *Bothriochloa insculpta*.
- A 2,3-DHP infusion treatment comprising the control diet plus 0.04% body weight (BW) 2,3-DHP, administered as an oral drench incrementally, 4 times a day. The 2,3-DHP was purchased as 2,3-dihydroxypyridine (95% pure) from Sigma-Aldrich®.
- Two levels of *Leucaena leucocephala* cv. Cunningham (leucaena) in diet: 50% leucaena and 50% local grasses as above; and 100% leucaena. These treatments

were designed to provide a comparison of naturally occurring 2,3-DHP with infused 2,3-DHP.

In the supplementary study, an additional 4 goats per treatment (average starting weight 14.0 ± 2.5 kg (±s.d.)) were given a mineral treatment comprising 0.04% BW Iron(II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) as an oral drench given incrementally, 4 times a day. This study, with a total of 32 goats, was conducted for the first 3 of the 7 weeks of the primary experiment.

There was a 5-day preliminary period that preceded the commencement of the experiment during which animals were gradually introduced to their full treatments, increasing incrementally every 2 days. All goats were originally from local villages and had previously consumed both leucaena and gliricidia. The trial was sanctioned under animal ethics approval # AFS/160/12/ACAIR (Appendix 3).

Table 7.1: Diet allocations for treatments applied in experiment 1.

*, Supplementary study

Treatment	*n*	Period of study (wks)	gliricidia in diet (%)	leucaena in diet (%)	local grasses in diet (%)	BW $FeSO_4 \cdot 7H_2O$ (%)	BW 2,3-DHP (%)
Gliridia-50 (ctrl)	4	7	50	0	50	0	0
±minerals	(4+4)*	(3)	50	0	50	0.04	0.04
Gliricidia-50+DHP	4	7	50	0	50	0	0
±minerals	(4+4)*	(3)	50	0	50	0.04	0.04
Leucaena-50	4	7	0	50	50	0	0
±minerals	(4+4)*	(3)	0	50	50	0.04	0.04
Leucaena-100	4	7	0	100	0	0	0
±minerals	(4+4)*	(3)	0	100	0	0.04	0.04

7.2.1 Urinary DHP measurements

Urine was collected from under each individual pen in a collection container. Pens had a slatted floor, with a mesh screen underneath to collect faeces allowing urine to pass through for collection. A 24-hour bulked urine sample was obtained weekly; from this a 9.5 mL sub-sample was extracted and stored acidified with concentrated hydrochloride acid (HCl). Samples were exported to Australia for analysis of DHP concentration using high performance liquid chromatography (HPLC) as described by Dalzell *et al.* (2012) with a corrected mobile phase of 25 mM ammonium dihydrogen phosphate.

The quantification of conjugated DHP was estimated from the area under a related peak of 2,3-DHP with a reduced retention time. This peak has previously been identified as conjugated DHP (Halliday *et al.* 2014b) and is discussed in Chapter 8.

7.2.2 Blood

Weekly blood serum samples were collected via jugular venipuncture into red-capped evacuated tubes (Benton Dickinson Vacutainer systems) with clot-activator using a BD Vacutainer Eclipse blood collection needle. Weekly blood plasma samples were also collected using royal-blue-capped BD Vacutainer® glass sterile tubes with Sodium heparin and 158 USP units as an anticoagulant. Tubes were inverted 5–8 times and immediately chilled. All samples were centrifuged at 1300 RCF for 10 minutes; the serum tubes were spun after clot activation. The serum and plasma samples were then transferred into polypropylene tubes and stored frozen (<−20ºC) prior to analysis.

Samples from weeks 4 and 6 were analysed by QML Pathology Vetnostics Service. Plasma samples were analysed for Fe concentration, according to the ferrozine method of Carter (1971) without the deproteinisation protocol, and Zn concentration according to the method of (Homsher and Zak 1985) using the 5-Br-PAPS protocol; quantification was performed on a Cobas 8000 biochemistry analyser (Roche). Serum samples were analysed for alanine aminotransferase (ALT) according to the recommendations of the expert panel on enzymes of the international federation of clinical chemistry (IFCC) (Bergmeyer 1980), however, without pyridoxal phosphate activation.

Experiment 2

Experiment 2 followed directly after experiment 1 and consisted of a 10-week trial period in which 20 kacang goats from experiment 1 (average starting weight 15.0 ± 3.4 kg (±s.d.)) were assigned to new treatments (Table 7.2). The control treatments were adjusted to include 100% gliricidia, to match the continuing 100% leucaena treatments. The mineral supplement was given orally 4-times per day at a total rate of 0.04% BW iron(II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$). There were 5 replications of each treatment. For logistical reasons, it was not possible to collect urine samples in experiment 2.

Table 7.2: Diet allocations for treatments applied in experiment 2.

Treatment	*n*	Percentage of gliricidia in diet	Percentage of leucaena in diet	Percentage of $FeSO_4 \cdot 7H_2O$ in diet (%BW)
Gliricidia-100	5	100	0	0
Gliricidia-100+M	5	100	0	0.04
Leucaena-100	5	0	100	0
Leucaena-100+M	5	0	100	0.04

Both experiments

Total dry matter (DM) was offered at a minimum of 25 g DM/kg LW.day, increasing as required to ensure a 10% refusal of feed offered. Rations were determined weekly after animal weighing. The experiments were conducted in an existing goat feeding shed ('kandang'), modified to house individual goats in separate feeding pens in a randomised complete block design (RCBD) with 4 and 5 replications in experiment 1 and 2, respectively. Each feed type was offered separately, with minerals delivered as an oral drench dissolved in 20 mL water. The 2,3-DHP was also delivered orally as a suspension in 20 mL water and the 2,3-DHP plus minerals treatment was delivered as a combined solution. Gliricidia and leucaena only animals were given a 20 mL water only drench to harmonise handling procedures.

Liveweight measurements were recorded weekly and prior to the daily allocation of feed and water, using a Dunlop 50 kg scale. Scales were calibrated with a standard weight prior to commencement of weighing and periodically throughout.

Legume forages were harvested fresh daily and consisted of fresh stripped leaf material only. All animals had previously consumed leucaena, gliricidia, and local grasses intermittently before entering the experiment. The local grasses were primarily *Bothriochloa insculpta* and were fed fresh chopped using a small petrol-powered chaff-cutter or were hand-chopped with a machete. The animals were selected from Sumlili village as previous samplings (Chapter 6) had identified that goats consuming leucaena in that area had high urinary DHP levels. This experiment was sanctioned under animal ethics approval # AFS/160/12/ACIAR.

7.2.3 Daily dry matter intake

Dry matter intake (DMI) of individual feed types was measured daily with weekly treatment mean values calculated from all animals within replicates. The dry matter of each feed

offered and refused was estimated by drying a sub-sample to constant weight in a portable wood-fired oven. The content of nitrogen (N) in samples was determined using a LECO TruSpec CHN analyser (Matejovic 1995) and neutral detergent fibre (NDF) and acid detergent fibre (ADF) were measured according to the method described in Van Soest *et al.* (1991).

7.2.4 Statistical analyses

Data were analysed using Microsoft Excel computer software (©2016, Microsoft, Redmond, WA, USA) and using Minitab® statistical software 17 (©2010, Minitab Inc., State College, PA, USA).

Repeated observations of weekly DMI and DHP levels in experiment 1 were analysed with a General Linear Model (GLM) ANOVA using a split-plot design (with treatment as the main-plot and time as the sub-plot; replications as a random factor) with pairwise comparisons using Fishers least significant difference (LSD) method. A GLM ANOVA was undertaken to compare animal LWG in experiment 1, with pairwise comparisons using Fishers LSD method.

Animal plasma Fe and Zn in experiment 1, and DMI and LWG in experiment 2 were analysed as a 2-way ANOVA using a GLM including legume treatment and mineral supplementation interaction term. One-way ANOVA tests were further used to compare significant terms, with pairwise comparisons made using Fishers LSD method. Descriptive statistics were used to summarise DHP concentrations, proximate analysis of the feed types, DMI, and animal LWG.

A probability level of $P < 0.05$ was considered statistically significant, while a *P*-value between 0.05–0.10 was considered a moderate trend.

7.3 Results

Experiment 1

7.3.1 Intake

There were no significant differences in the mean total intake of animals offered 50% gliricidia in diet, indicating that infusion with 2,3-DHP did not reduce intake in comparison with the control treatment. However, intake of animals offered 50% leucaena (leucaena-50) was greater than that of animals offered gliricidia ($P < 0.01$). The intake of animals offered 100% leucaena was greater than other treatments and almost double that of animals offered 50% gliricidia, due to the greater intake of legume compared to the legume plus grass diets (Table 7.3). Thus, there was no negative effect of DHP on levels of intake, whether administered as oral 2,3-DHP directly or via the breakdown of mimosine to 3,4-DHP and 2,3-DHP from leucaena in the diet.

The effective proportion of legume in diet of the legume plus grass treatments was 62% for gliricidia and 74% for leucaena, again indicating a preference for legume over grasses and a higher preference for leucaena over gliricidia. The treatment means for all weeks were pooled over the 7-week period; no significant week × treatment interaction ($P = 0.12$) was observed.

Table 7.3: Liveweight gain (LWG), dry matter intake (DMI) and urinary hydroxypyridone (DHP) levels of *Capra hircus* goats in response to diets of gliricidia and leucaena, infused with DHP (Experiment 1) or supplemented with $FeSO_4$ mineral salts (Experiment 2).

Means within a column with differing subscripts denote significant differences at the 5% significance level.

Expt	Length (weeks)	Treatment	LWG (g/day)	Leucaena DMI (g/day)	Gliricidia DMI (g/day)	Grass DMI (g/day)	Total DMI (g/day)	Total DMI (g DM/kg LW.day)	Proportion legume (%)	Mean 3,4-DHP (mg/L.day)	Mean 2,3-DHP (mg/L.day)	Proportion of 2,3-DHP remaining conjugated
1	7	Gliricidia-50	$15_{ab} \pm 3$	0	196 ± 8	137 ± 8	334 ± 16	$28_a \pm 1$	60 ± 1	$8_a \pm 3$	$64_a \pm 22$	–
		Gliricidia-50+DHP	$2_a \pm 2$	0	215 ± 10	127 ± 7	343 ± 16	$26_a \pm 1$	63 ± 1	$20_a \pm 10$	$426_b \pm 74$	$25\%_a$
		Leucaena-50	$23_b \pm 3$	287 ± 15	0	103 ± 9	391 ± 22	$31_b \pm 1$	74 ± 1	$160_b \pm 39$	$636_{bc} \pm 97$	$22\%_a$
		Leucaena-100	$41_c \pm 8$	756 ± 36	0	0	756 ± 36	$54_c \pm 2$	100	$86_c \pm 19$	$772_c \pm 112$	$36\%_a$
2	10	Gliricidia-100	$22_a \pm 9$	0	511 ± 20	0	511 ± 20	$37_a \pm 1$	100	–	–	–
		Gliricidia-100+M	$18_a \pm 6$	0	494 ± 14	0	494 ± 14	$37_a \pm 1$	100	–	–	–
		Leucaena-100	$44_b \pm 8$	1006 ± 25	0	0	1006 ± 25	$53_b \pm 1$	100	–	–	–
		Leucaena-100+M	$29_{ab} \pm 3$	984 ± 22	0	0	984 ± 22	$53_b \pm 1$	100	–	–	–

7.3.2 Liveweight gain

The mean daily liveweight gain (LWG) of animals receiving gliricidia-50 and leucaena-50 was similar over the 7-week period. Animals receiving the gliricidia-50+DHP diet had the lowest weight gain (Table 7.3), while the highest mean LWG was achieved by animals fed 100% leucaena and was approximately double the mean of animals fed treatments of leucaena-50 or gliricidia-50 (P = 0.01). A regression of LWG with DMI displayed a significant trend (LWG = −12.6 + 0.945 × DMI; P = 0.01; R^2 = 52%), as was expected.

The LWG of goats at conclusion of the 3-week supplementation study was reduced in all treatments supplemented with iron(II) sulphate (Table 7.4).

Table 7.4: Liveweight gain (LWG) after the 3-week supplementary study of experiment 1 involving *Capra hircus* goats in response to diets of gliricidia and leucaena, infused with DHP and supplemented with $FeSO_4$ mineral salts.

Treatment	LWG (g/day)
Gliricidia-50	-0.23
Gliricidia-50+M	-0.50
Gliricidia-50+DHP	0.20
Gliricidia-50+DHP+M	-0.35
Leucaena-50	0.60
Leucaena-50+M	-0.40
Leucaena-100	0.75
Leucaena-100+M	0.35

7.3.3 Urinary DHP excretions

The mean weekly urinary 3,4-DHP and 2,3-DHP levels were highly variable, due in part due to their relatively low concentrations, with no significant week × treatment interaction (P = 0.15). Thus, the treatment means of all weeks were pooled over the 7-week period. As expected, the mean daily urinary 3,4-DHP excretions were substantially lower than 2,3-DHP excretions, being present at levels between 5–25% of 2,3-DHP. The highest level of 3,4-DHP was detected in the leucaena-50 treatment, almost twice that of animals receiving double the amount of leucaena (leucaena-100 treatment) (Table 7.3). The levels of 2,3-DHP were not significantly different between leucaena treatments, although they were higher in the leucaena-100 compared to the gliricidia-50+DHP treatment (P = 0.03). The percentage of 2,3-DHP remaining conjugated after hydrolysis treatment was consistent among treatments.

Low levels of DHP were detected in the collections from animals receiving the control (gliricidia-50) treatments, despite not receiving any leucaena or DHP. This was indicative

of a small level of contamination during urine collection. However, levels were negligible and did not greatly affect treatment means.

In the 3-week supplementary investigation, the mean levels of total DHP excreted (combined 3,4-DHP and 2,3-DHP) when Fe(II) mineral supplementation was administered were reduced by ~40% for gliricidia-50+M and leucaena-100+M, and by ~70% for gliricidia-50+DHP+M and leucaena-50+M treatments compared to un-supplemented treatments (Table 7.5).

Table 7.5: Urinary hydroxypyridone (DHP) levels of the supplementary study of experiment 1 involving *Capra hircus* goats in response to diets of gliricidia and leucaena, infused with DHP and supplemented with $FeSO_4$ mineral salts.

Experiment	Length (weeks)	Treatment	Mean 3,4-DHP (mg/L.day)	Mean 2,3-DHP (mg/L.day)	proportion of 2,3-DHP conjugated
$1_{supplementary}$	3	Gliricidia-50+M	6	10	–
		Gliricidia-50+DHP+M	0	211	30%
		Leucaena-50+M	56	288	14%
		Leucaena-100+M	49	139	17%

7.3.4 Blood constituents

Plasma Iron

The mean plasma Fe level of all samples was 1.94 ± 0.14 mg/L. There were no significant differences in mean plasma Fe levels between week 4 or 6 (P = 0.13) or due to the 4 treatments offered (P = 0.27); week 4 included the final collection from goats receiving mineral supplementation (total 8 treatments) which had no effect on plasma Fe levels.

Plasma Zinc

There was a positive effect on plasma Zn with increasing percentage of leucaena in diet (P < 0.01), and a negative effect due to Fe(II) mineral supplementation (P < 0.01), with levels highest in leucaena fed goats not receiving Fe supplementation (P < 0.01) (Table 7.6). The levels of plasma Zn in all treatments, including control goats, were below normal levels reported by Mills (1978) of 0.9–1.2 mg/L for calves and lambs. There was no significant difference in plasma Zn levels between week 4 or 6 (P = 0.55).

Table 7.6: Mean plasma Zn levels collected in weeks 4 and 6 from *Capra hircus* goats in response to diets of gliricidia infused with DHP or leucaena, with and without Fe mineral supplementation.

Means with differing subscripts denote significant differences at the 5% significance level

Treatment	*n*	Mean plasma Zn (mg/L)
Gliricidia-50	4	$0.15_a \pm 0.05$
Gliricidia-50+M	4	$0.18_a \pm 0.05$
Gliricidia-50+DHP	4	$0.15_a \pm 0.05$
Gliricidia-50+DHP+M	4	$0.20_a \pm 0.10$
Leucaena-50	4	$0.38_b \pm 0.05$
Leucaena-50+M	4	$0.20_a \pm 0.07$
Leucaena-100	4	$0.58_c \pm 0.05$
Leucaena-100+M	4	$0.13_a \pm 0.03$

Serum ALT

The mean serum alanine aminotransferase (ALT) level of all goats was 15.4 ± 2.0 U/L. There were no significant differences between week 4 or 6 (P = 0.70) or between treatments (P = 0.26).

Experiment 2

7.3.5 Intake

The mean daily intake of animals consuming leucaena diets (leucaena-100 and leucaena-100+M) was significantly higher ($P < 0.01$) at approximately double that of animals on gliricidia diets (gliricidia-100 and gliricidia-100+M) (Table 7.3). The weekly trends in intake were consistent among treatments with no week × legume ($P = 0.69$), or week × minerals interaction ($P = 0.99$). Thus, the treatment means were pooled over the 10 weeks.

There was no significant effect of mineral supplementation on DMI ($P = 0.54$), or a significant interaction between mineral supplementation and legume offered ($P = 0.80$).

7.3.6 Liveweight gain

Mean daily liveweight gains were calculated for each treatment over the 10-week period (Table 7.3). Animals on diets containing 100% leucaena only (leucaena-100) achieved LWGs approximately double those of animals on 100% gliricidia diets (gliricidia-100 and gliricidia-100+M) ($P = 0.02$), reflecting the similar trend in intake levels. There was no significant effect on LWG due to supplementation with minerals ($P = 0.14$) nor a significant interaction of mineral supplementation with legume offered ($P = 0.36$).

Both experiments

7.3.7 Feed chemical composition

The crude protein content was highest in the fresh cut leucaena leaf material. The high voluntary intake and preference for leucaena can also be attributed to the low NDF and ADF values of the forage (Table 7.7). While leucaena leaf contained the lowest levels of both Fe and Zn (Table 7.7), the Fe and Zn levels of all feed types exceeded adequate levels for plant health (>44 mg Fe/kg DM and >13 mg Zn/kg DM) (H. M. Shelton, personal communication).

Table 7.7: Chemical composition (g/kg DM) of feed types offered to *Capra hircus* goats.

DMD: dry matter digestibility; GE: gross energy; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre

Parameter	Leucaena	Gliricidia	Local grasses
CP (g/kg DM)	223 ± 8	153 ± 8	58 ± 5
NDF (g/kg DM)	447 ± 22	498 ± 11	699 ± 7
ADF (total) (g/kg DM)	365 ± 21	410 ± 19	456 ± 6
Fe (mg/kg)	88 ± 7	107 ± 17	422 ± 80
Zn (mg/kg)	14 ± 1	18 ± 3	34 ± 2

7.4 Discussion

Much has been learned in the last 10 years of research regarding leucaena toxicity, contradicting the original hypothesis that bacterial degradation by *Synergistes jonesii* was the principal pathway of DHP degradation. This has led to an updated/alternate hypothesis: that *Synergistes jonesii* is not capable of complete detoxification of DHP in ruminants on high leucaena diets and other physiological and/or metabolic pathways are operating to detoxify DHP (Halliday *et al.* 2013; Halliday *et al.* 2014b). The findings from this experiment support this hypothesis and are now discussed in detail with respect to the key objectives.

7.4.1 Toxicity response to infused 2,3-DHP administered orally

The first objective of this study was to investigate the toxicity of 2,3-DHP as it is persistently present in high concentrations in animals maintained on leucaena diets worldwide (Halliday *et al.* 2014b). The infusion rate of 0.04% BW 2,3-DHP in this study resulted in urinary levels of 2,3-DHP comparable with the 50% leucaena diet with no observable signs of acute toxicity from any treatment. In comparison, in a similar study by McSweeney *et al.* (1984), when 2,3-DHP was administered continuously via a rumen fistula at the same rate of 0.04% BW, sheep stopped eating after 7 days. And in an Indian study, the single intraruminal dose of 0.017% BW 2,3-DHP administered by Puchała *et al.* (1995) was fatal within 24 hours in Angora goats.

Although 2,3-DHP toxicity in ruminants is comparatively less studied, it was assumed to be similarly toxic as 3,4-DHP (D'Mello 1992; Paul 2000; Ghosh *et al.* 2008), based on the assumption that it possessed similar goitrogenic effects (Jones *et al.* 1978; Christie *et al.* 1979; Hegarty *et al.* 1979; Jones 1979; Lee *et al.* 1980; Megarrity and Jones 1983; Jones *et al.* 1985a; Lowry *et al.* 1985; Dalzell *et al.* 2012). Studies involving the administration of the isomer 3,4-DHP (0.02% BW) were fatally toxic in rats and monkeys, and clinical signs included organ atrophy, and damage to bone marrow (Berdoukas *et al.* 1993; Hoffbrand and Wonke 1997; Porter 1997; Kwiatkowski 2008). These studies notably did not observe any goitrogenic effects of 3,4-DHP. In other trials, the development of goitre itself has been shown to be caused by metal deficiencies, with compromised thyroid function in Fe deficient rats (Smith *et al.* 1992), depressed thyroxin levels after 42 days in Zn deficient rats (Lukaski *et al.* 1992), and Zn deficiency via urinary excretion correlated with hypothyroidism in human patients (Nishi *et al.* 1980).

Metal deficiencies of Fe, Cu, Zn also explain many of the other signs of toxicosis, including hair loss in rats and mice (Cunningham 1932; Hashiguchi and Takahashi 1977) (Day and Skidmore 1947; Smith and Ellis 1947; Hashiguchi and Takahashi 1977; Stunzi *et al.* 1980) and DNA inhibition (Tsai and Ling 1971). Considerable work has also been conducted in the pharmacological field, with hydroxypyridones used as orally active metal chelators in patients with iron overload (Santos 2008; Santos *et al.* 2012; Lachowicz *et al.* 2015). Examples include hereditary haemochromatosis (Sooriyaarachchi and Gailer 2010) which affects approximately 1 in 300 people of European background (Bridges 2001) and thalassaemia intermedia (Zhou *et al.* 2006; Lachowicz *et al.* 2015). The reported negative side effects of continued exposure to DHP in chelation therapy are well documented and make no mention of a reduction of thyroid hormones, rather, are related to the disruption of homeostasis of essential metals (Sooriyaarachchi and Gailer 2010), especially Zn (Megarrity and Jones 1983; Barman Balfour and Foster 1999; Hoffbrand 2005; Crisponi and Remelli 2008). This affected a wide range of vital metalloenzymes (Berdoukas *et al.* 1993; Hider 1995; Hoffbrand 2005) including superoxidase dismutases, such as succinate dehydrogenase (Tsai 1961) and alkaline phosphatase (El-Harith *et al.* 1981), both of which require Zn, Mg, and Cu.

The main toxic effect of DHP (in humans, rats, mice, monkeys, and ruminants) is now understood to be due to its action as a strong ligand, chelating essential metals *in vivo*. This occurs because although DHP is often described as a dihydroxypyridine, it actually exists as the keto-enol tautomer with the equilibrium strongly favouring the hydroxypyridone form at physiological pH (Scarrow *et al.* 1985).

The referral of hydroxypyridones as DHP in ruminant toxicology can be considered something of a misnomer, as many chemists have chosen more descriptively accurate abbreviations of what exists at physiological pH as 3-hydroxy-2(1H)-pyridone such as: 2,3-DOHP (Morais *et al.* 2006); H2pp (Nurchi *et al.* 2012); 3,2-Hopo (Scarrow *et al.* 1985); 3,2-HOPO (Harrington *et al.* 2010; Santos *et al.* 2012); and vhpo (Stunzi *et al.* 1979). Taking these into account, an abbreviation such as HPo would more descriptively represent the hydroxypyridone conformation. For consistency however, this thesis will continue to use the generally accepted abbreviation of DHP.

In hydroxypyridone form, the O substituent vicinal to the C-3 enol/hydroxyl (–OH) moiety, exists as a keto/carbonyl (=O) moiety (Figure 7.1a & b). This is due to the resulting stability of the zwitterionic canonical mesomer in this form, with the delocalisation of electrons from the N resulting in a high charge density over the *para* or *ortho* carbonyl group (in 3,4-DHP

and 2,3-DHP, respectively) (Figure 7.1c & d), which also has a greater bond strength (Cottrell 1958), and is further promoted by the aromatic resonance of the ring (Gardner and Katritzky 1957; Hider 1995; Santos 2002). The pyridinium ring N does not deprotonate in aqueous solution (Curtis *et al.* 1970; Stunzi *et al.* 1980).

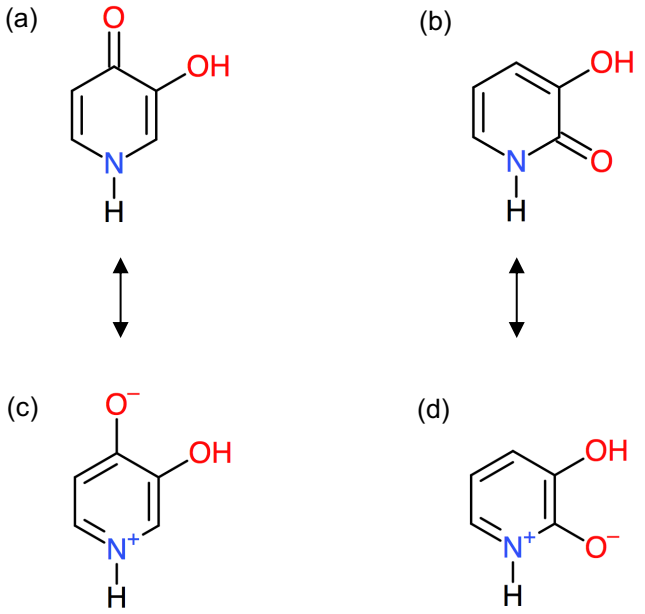

Figure 7.1: Structures of: (a) the 3-hydroxy-4(1*H*)-pyridone (3,4-DHP) tautomer in keto-enol form; (b) the 3-hydroxy-2(1*H*)-pyridone (2,3-DHP) tautomer in keto-enol form; (c) the zwitterionic mesomer of 3,4-DHP; and (d) the zwitterionic mesomer of 2,3-DHP. Adapted from Curtis and Atkinson (1972), Stunzi *et al.* (1980) and Scarrow *et al.* (1985).

The resultant hydroxo-carbonyl moiety on the pyridine ring of hydroxypyridone makes it a strong bidentate (2 binding points) ligand (Gardner and Katritzky 1957; Goel *et al.* 1970; Stunzi *et al.* 1980; B. Di Marco *et al.* 1999; Flora and Pachauri 2010). Hydroxypyridones, including 3,4-DHP and 2,3-DHP, bind metals via ionic bonds with the oxyanion (deprotonated 3-hydroxyl) (Goel *et al.* 1970; Vovk *et al.* 2003) and coordinate bonds with an *ortho* electronegative carbonyl moiety (Scarrow *et al.* 1985). At physiological pH, this forms a 2:1 ligand:metal ratio with bivalent cations in four-coordinate geometries using x-ray crystallography (Clarke and Martell 1992; Flora and Pachauri 2010).

The crystal structure of 2,3-DHP has been confirmed with trivalent Al(III) and Fe(III) complexes binding in 3:1 ligand:metal ratios (Scarrow *et al.* 1985; B. Di Marco *et al.* 1999; Nurchi *et al.* 2012) with X-ray diffraction data confirming the bond through the (*O*,*O*) complex (Streater *et al.* 1990; Vovk *et al.* 2003) (Figure 7.2). The 1,2-dimethylated 3,4-DHP (3-hydroxy-1,2-dimethyl-4-pyridone), pharmacologically used as iron chelator and

branded as Deferiprone (DFP) (Galanello 2007) also forms chelate bonds via the (*O*,*O*) complex (Xiao *et al.* 1992; Melchior *et al.* 2001; Sooriyaarachchi and Gailer 2010; Kaviani *et al.* 2016).

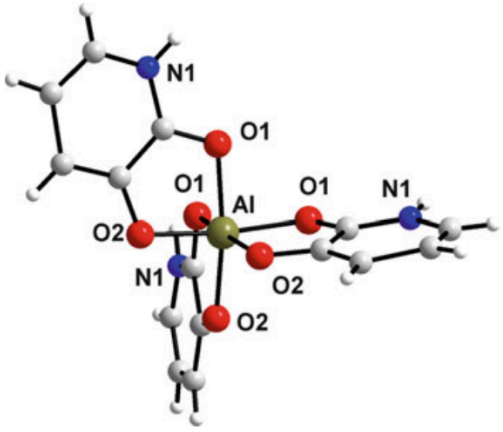

Figure 7.2: Crystal structure of the Al(III):2,3-DHP$_3$ complex, catalogued in the Cambridge Structural Database (CSD) as CAQDON, source Nurchi *et al.* (2012). Scarrow *et al.* (1985) and B. Di Marco *et al.* (1999) reported identical structures with Fe(III) and Al(III), respectively.

Although both isomers of DHP bind in the same fashion, the strength of these two ligands, measured by the stability constants for protonation of the chelating hydroxyl moiety (pKa1) (Curtis and Atkinson 1972; Scarrow *et al.* 1985), is markedly different. The pKa1 of 2,3-DHP (8.7) is substantially lower than 3,4-DHP with a pKa1 of between 9.5–9.9 (Dobbin *et al.* 1993; Santos 2002). The reason for the decreased ligand strength of 2,3-DHP, is due to the arrangement of the electronegative carbonyl moiety at the C-2 position, which places it nearer the positive pyridinium ring-N (Santos 2002). The reduced electron density of the coordinating hydroxyl/carbonyl (*O*,*O*) moiety (Santos *et al.* 2012) and the electrostatic repulsions between the positive charge of the pyridinium N have an inductive destabilising effect on the chelate (B. Di Marco *et al.* 1999) resulting in a decreased binding strength with metal cations (Santos 2002). The resulting affinity of 2,3-DHP to bind with essential elements such as Cu(II), Zn(II), Mg(II) and Ca(II) at physiological pH (~7.4) is reduced by factors of 30, 10, 4, and 2, respectively in comparison to 3,4-DHP (Stunzi *et al.* 1980; Dobbin *et al.* 1993). This weaker chelating ability of 2,3-DHP (Spinner and White 1966; Stunzi *et al.* 1980; Streater *et al.* 1990; Dobbin *et al.* 1993) has the result of significantly reducing its toxicity *in vivo*. The reduction in ligand strength has even precluded it as a ligand for iron chelation therapy (Santos 2002).

The results of this study support the observation that 2,3-DHP has a reduced toxicity in comparison to 3,4-DHP, as intake of leucaena was not negatively affected in

unsupplemented 2,3-DHP-infused goats over the 7-week period and there was no significant impact on LWG in comparison to control gliricidia-50 animals. The LWG of gliricidia-50+DHP animals was lower in comparison to the leucaena-50 treatment, with an equivalent DHP intake, although this effect was confounded with the different legume diet. Another possible contributing factor for the lack of toxicosis was that the goats in this study were already adapted to eating leucaena, unlike the naïve animals in the experiments of McSweeney *et al.* (1984) and Puchała *et al.* (1995). This previous adaption to leucaena may have afforded them tolerance of 2,3-DHP, a common phenomenon observed in productive ruminants maintained for long periods on leucaena diets despite persistent high levels of 2,3-DHP excreted (Halliday *et al.* 2014b). It is noted that even the Hawaiian goats, assumed to be protected by the presence of the ruminal bacterium *S. jonesii*, in the seminal study of Jones and Megarrity (1983), were excreting DHP as the 2,3-DHP isomer, without negative effects.

7.4.2 The effects of mineral supplementation on detoxifying DHP

The objective of the second experiment was to investigate the ability of mineral supplementation with iron(II) sulphate ($FeSO_4$) to promote the intraruminal chelation of DHP as a prophylactic protective mechanism. Supplementation was intended to exploit the high affinity of DHP to bind with Fe cations, forming a stable chelate, reducing the toxicity and increasing urinary clearance of the chelated DHP (Puchała *et al.* 1995; Galanello 2007; Flora and Pachauri 2010).

Surprisingly, the findings of this study did not support the hypothesis of increased DHP excretion. When a solution of iron(II) sulphate was administered in the short-term study in experiment 1, there was a slight reduction of 2,3-DHP excreted with the greatest effect in goats receiving 100% leucaena and in animals receiving 2,3-DHP orally (Table 7.5). In other work, the combination of Fe(II), Cu(II), and Zn(II) (Jones *et al.* 1978) and Fe(II), Mg(II), and Zn(II) (Puchała *et al.* 1995) mineral salts doubled urinary clearance compared to un-supplemented goats infused with 2,3-DHP. While the use of multiple metal supplements will aid in correction of deficiencies of these elements, that was not the objective of this study. A single element supplement was employed to isolate the effects of intraruminal detoxification of DHP via chelation. The very low levels of Zn in goats fed gliricidia, as well as those fed leucaena with Fe supplementation, was unexpected, and did not appear associated with DHP toxicity. Also, although the stability constant of Fe(II) when binding with DHP is the lowest of the bivalent transition metals (12.1), the values are

relatively similar. It is therefore unlikely that an alternate metal supplement for promoting chelation (such as Cu(II) or Zn(II)) would have affected the outcome.

The addition of the Fe(II) treatment in experiment 2 also did not improve animal performance as hypothesised. Un-supplemented animals achieved the same intake as those receiving the Fe(II) treatment and there was a trend of reduced LWG in 100% leucaena-fed goats receiving Fe(II). Mineral supplementation with Fe(II) (Ross and Springhall 1963), or in combination with other bivalent elements (Jones *et al.* 1978; Puchała *et al.* 1995) has previously been shown to increase DMI, LWG, and decrease clinical signs of DHP toxicosis (Tsai and Ling 1974; Jones *et al.* 1978; Puchała *et al.* 1995). Possible reasons for the lack of response include: (a) alternate coping mechanisms preventing the development of DHP toxicity in any treatment; and, (b) a possible contraindication of the Fe(II) supplementation treatment. Although not quantified, there was indication that the Fe(II), delivered as an aqueous solution of $FeSO_4$, appeared to be partially oxidised to Fe(III) from dissolved oxygen in the water. This was observed as a yellow colour in solution with water, indicative of Fe(III) hydroxide precipitate. The solution of Fe(II) sulphate and 2,3-DHP also immediately turned a purple colour, indicative of oxidisation to the Fe(III) ion (Howlin *et al.* 1982; Scarrow *et al.* 1985). Once precipitated out of solution, Fe(III) hydroxide would be incapable of binding 2,3-DHP intraruminally, when delivered orally.

Despite the similar DMI and LWG of goats both receiving the Fe(II) supplementation treatment and those without, there were no observed negative effects of the Fe(II) sulphate solution on these parameters in experiment 2. However, during the supplementary mineral study within the first 3 weeks of experiment 1, albeit a short period to assess LWG differences, there was an observed trend in loss of LW in all treatments by −27 ± 8 g/day when supplemented with Fe(II) sulphate (Table 7.4).

7.4.3 Impact of DHP on LWG

There was no indication of subclinical toxicity (depressed LWG) in goats in relation to DHP-induced mineral deficiencies. The unremarkable mean plasma Fe levels of goats in all treatments (1.94 mg/L) was within the published normal range for small ruminants (1.66–2.22 mg/L) (Constable *et al.* 2017). This was maintained by adequate dietary Fe levels in the diets offered (88–224 mg/kg DM); within the nutritional range for goats (35–500 mg/kg DM) (Hart 2008).

Plasma Zn levels were, however, below adequate (0.8 mg/L) (Mills 1978) in all treatments; although goats fed diets of leucaena without Fe mineral supplement had the highest plasma Zn levels (Table 7.6). The addition of Fe(II) sulphate reduced plasma Zn levels in these animals to levels similar to those of goats on gliricidia-based diets; Fe(II) sulphate had no effect on the already low plasma Zn levels in gliricidia-fed goats. It is possible that Zn deficiency in leucaena-fed goats supplemented with Fe(II) sulphate was due to excess Fe, not involved in chelation with DHP, decreasing the absorption of dietary Zn (Hart 2008); however, it is not clear why plasma Zn levels were low in control animals. Mills (1978) reported clinical signs of Zn deficiency occurred at serum levels <0.8 mg/L and loss of LW at levels <0.4 mg/L. Although plasma Zn levels were below 0.6 mg/L in all treatments, there were no apparent clinical signs of Zn deficiency. Although the Zn levels in leucaena were adequate for plant growth, the percentage of Zn in diet (14 mg Zn/kg DM) in the leucaena-100 treatments was below required levels for goats (40–500 mg Zn/kg DM) (Hart 2008). While the signs of DHP toxicity are associated with essential element deficiencies, including Zn (Megarrity and Jones 1983), the low levels of Zn in this study did not appear associated with DHP. On the contrary, high leucaena diets promoted high serum Zn levels and high liveweight gains. This occurred, despite high levels of 2,3-DHP remaining undegraded.

7.4.4 Evidence of conjugation occurring

It has been suggested (Puchała *et al.* 1995) that chelation may be necessary for efficient excretion of DHP, as the increased polarity of the chelate prevents reabsorption in the kidneys. While an increase in DHP excretion in association with Fe mineral supplementation was not observed, a similar effect is achieved when DHP is bound as a conjugate (Galanello 2007). In fact, the chelatory ability of DHP is reduced when conjugated (Singh *et al.* 1992; Crisponi and Remelli 2008). As there was evidence of considerable conjugation occurring, this also may have negated the effectiveness of the mineral treatment. This pathway is now discussed.

Despite samples in this study undergoing acid hydrolysis at collection, not all DHP was hydrolysed to the free form, as indicated by HPLC results. In animals fed 100% leucaena during the 7-weeks of experiment 1, up to 42% of DHP excreted remained conjugated after acid hydrolysis; animals fed lower levels of leucaena/DHP had a lower level of conjugation of 28%. While the total amount of DHP conjugated prior to hydrolysis was not measured, other studies have reported up to 90% of DHP to be excreted as a conjugate (Singh *et al.* 1992; Flora and Pachauri 2010) preventing toxicosis.

The underlying principle of conjugation is similar to that of chelation: binding of the hydroxyl site reduces the affinity of the molecule to act as a ligand, and therefore limits depletion of metal ions (Singh *et al.* 1992). Once conjugated, DHP is rapidly eliminated from the body within 1–2 hours (Galanello 2007; Flora and Pachauri 2010; Sooriyaarachchi and Gailer 2010). The glucuronic acid complex is the most common conjugation pathway (glucuronidation) in mammals and is a non-specific mechanism for detoxifying a variety of xenobiotic compounds (Gupta and Akbar 1993; Sahlu *et al.* 1995; Galanello 2007; Crisponi and Remelli 2008). The identification of the conjugate is described in detail in Chapter 8, whereby specialised collection methodologies were utilised to preserve the conjugated DHP from hydrolysis.

Despite conjugation primarily occurring in the liver (Gupta and Atreja 1998), the persistent high levels of 2,3-DHP entering circulation did not appear to affect liver health. Elevated serum alanine aminotransferase (ALT) levels are indicative of liver damage and have been observed in patients receiving DHP chelation therapy (Barman Balfour and Foster 1999; Cohen *et al.* 2000; Ha *et al.* 2006; Roberts *et al.* 2007; Fisher *et al.* 2013). However, ALT levels were not elevated in goats on any treatment and were within normal levels for small ruminants (5–20 U/L) (Constable *et al.* 2017). Similar findings (~12 U/L) were recorded in the work of Girdhar *et al.* (1991) where no significant increase was recorded in leucaena-fed goats over an 8-month period.

It is entirely possible that conjugation has always been an important mechanism of detoxification. Much like the underappreciated significance of 2,3-DHP by Jones and Megarrity (1983), the methodologies for preserving DHP were not suitable for accurately quantifying conjugated DHP due to acidification of the samples prior to analysis (Jones and Lowry 1984). This acidification step hydrolysed conjugated DHP to the free (toxic) form. However, it is now apparent that considerable amounts of residual conjugated DHP commonly remain. Even the improved methodologies for hydrolysing DHP (Graham *et al.* 2014) have not achieved complete hydrolysis of the conjugate (Halliday *et al.* 2014b). It is therefore likely that the conclusion of Jones and Lowry (1984), that detoxification of DHP occurred via microbial degradation, underestimated the importance of the conjugation pathway.

7.4.5 Low levels of DHP in 100% leucaena-fed goats

The level of total DHP excreted when goats were administered 2,3-DHP orally at a rate of 0.04% BW were similar to that of goats receiving 50% leucaena in diet, which contained an equivalent level of DHP intake. However, a surprising result was that animals

consuming a 100% leucaena diet did not have significantly higher levels of DHP in urine. This is in contrast with results obtained from similar goats from the same farm in Sumlili during sampling in 2011 (Chapter 6) where mean 2,3-DHP levels reached 4980 mg/L 2,3-DHP.

Other studies have similarly shown a variable relationship between amount of leucaena ingested and levels of DHP in urine. The study of Tangendjaja *et al.* (1985) found that DHP levels in Indonesian goats fed 100% leucaena declined from high levels (7000 mg/L) to approximately 300 mg/L after 3 weeks of feeding. These researchers did not discriminate as to which isomer of DHP was detected. In the feeding trial in Chapter 5, levels of total DHP declined in undrenched leucaena-fed steers over a 6-week period. This effect was most pronounced in the 100% leucaena treatment, and coincided with an increase in leucaena intake. The low levels of DHP in urine of goats fed 100% leucaena in these studies and the current experiment suggest possible evidence for loss of DHP through metabolism with indigenous microbes, including *S. jonesii*. Nevertheless, the continued presence of DHP in animals known to have *S. jonesii* (Dalzell *et al.* 2012; Halliday *et al.* 2014c) (Chapter 5) suggest bacterial degradation alone is not sufficient to afford complete protection against DHP toxicity.

7.4.6 Leucaena is the most productive diet

Despite high urinary DHP excretions, the leucaena-fed goats showed no indication of reduced intake or LWG. Furthermore, 100% leucaena-fed goats outperformed all other treatments offered, demonstrating the high production potential of a high leucaena diet when animals are adapted. Intake and mean LWG of animals on 100% leucaena diets were up to 2-times greater than for animals fed 50% leucaena or 50% gliricidia combined with local grasses. These sole leucaena diets are commonly fed to goats, buffalo and Bali bulls in East Nusa Tenggara, including on the island of West Timor where this study was conducted. This is often out of necessity due to a lack of availability of other forages in the dry season (Dahlanuddin *et al.* 2014), however it is clear it also offers substantial production advantages.

The high productivity of 100% leucaena diets in this study is in strong contrast with the results of other feeding trials which have reported poor animal performance (Puchała *et al.* 1995; Ghosh *et al.* 2008) (Chapter 5). A major difference in this study was that the goats were already accustomed to consuming leucaena. Animals naïve to leucaena require an adaptation period of between 4–8 weeks during which intake is often reduced and clinical signs of toxicosis including alopecia are common (Mullenax 1963; Hegarty *et al.* 1964b).

Adaptation involves the proliferation of endogenous rumen microorganisms (Hegarty *et al.* 1964b) capable of degrading mimosine to DHP (Ghosh *et al.* 2007; O'Reagain *et al.* 2014), and the induction of enzymatic pathways involved in conjugation, primarily in the liver (Mohamed and Frye 2011). Once adapted, animals are capable of excellent performance on high leucaena diets (Panjaitan *et al.* 2014).

7.5 Conclusion

This study has highlighted the high value of leucaena as a forage, especially when fed at high levels. It has also demonstrated the efficacy of adapted animals to detoxify high levels of DHP, providing credible evidence that 100% leucaena diets can be safe and productive.

The first objective was to confirm the toxicity of 2,3-DHP. However, no clinical signs or a negative effect on intake were observed in any treatment. The infusion of 2,3-DHP to goats offered 50% gliricidia appeared to reduce LWG over the 7-week period in comparison to diets of gliricidia without 2,3-DHP, though the difference did not reach statistical significance. There were no differences in the leucaena-fed goats. Animals fed 100% leucaena diets outperformed those of all other treatments. The lack of toxicity was likely associated with the higher nutritive value of leucaena coupled with the detoxification and excretion of DHP in conjugated form, reducing the metal binding affinity and preventing potential deficiencies of essential elements.

The second objective was to investigate the impact of Fe(II) mineral supplementation on chelation of DHP as a mechanism to prevent toxicity. A reduction in toxicity of DHP with supplemented Fe(II) was not observed in this study. The notion of conjugation however, was identified as an important protective pathway. Recent studies, and the results of earlier chapters, have shown that although *S. jonesii* is part of the normal ruminal flora, it is rarely present in sufficient numbers to provide sole protection against toxicity (Padmanabha *et al.* 2014) (Chapter 5).

It is therefore concluded that the high leucaena diets used in this study were not toxic. The comparatively low levels of DHP in the urine of goats in the 100% leucaena treatment, along with the isomerisation of 3,4-DHP to 2,3-DHP (a weaker ligand and less toxic isomer), suggest an endemic *S. jonesii*, or possible other bacterial population, was likely responsible for some degradation of DHP. However, the high levels of remaining DHP (normally considered toxic (Dalzell *et al.* 2012)) were being detoxified by innate metabolic pathways, such as the conjugation in the liver. While the relative significance of each pathway is not definitively known, the presence of high quantities of DHP in urine without the accompanying toxicity signs, indicates hepatic detoxification as a major pathway.

As animals inherently possess the capacity to conjugate a variety of toxins, this eliminates the need for the labour-intensive practice of inoculation. This has the advantage of considerable savings for Australian graziers utilising leucaena pastures, if a similar

pathway exists in Australian cattle, and it greatly benefits smallholder farmers in developing countries where the supply chain and expertise to distribute and administer an inoculum does not exist.

The low plasma Zn levels in all animals indicated the need for a mineral supplement to complement the forage. While there was no indication of DHP-induced essential element deficiencies, mineral supplementation will promote a net positive essential element balance, especially during the mildly symptomatic adaptation phase, after animals are first introduced to leucaena.

Further research should focus on the conjugation pathways involved in detoxifying DHP, especially in animals fed 100% leucaena diets.

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Chapter 8 Longitudinal survey of Bali bulls (*Bos javanicus*) in eastern Indonesia and the prevalence of conjugation of hydroxypyridone as a detoxification mechanism

8.1 Introduction

The fattening of bulls for slaughter is of great importance to the profitability of farming enterprises of smallholder farmers in eastern Indonesia (Waldron 2016). The feeding of forage tree legumes (FTLs), especially *Leucaena leucocephala* (leucaena), in these cut-and-carry systems, is a vital contributor to the profitability of this system as it is a low-cost protein source and has greatly improved the nutrition of Indonesian smallholder cattle and reduced associated labour costs. The Balinese farmers in the hamlet of Jati Sari, within Rhee village on Sumbawa island, are a notable example of this phenomenon (Panjaitan *et al.* 2014). They have successfully fattened bulls on sole leucaena diets for over 20 years.

Despite the many excellent nutritional properties of leucaena, the presence of the toxin mimosine in plant tissue and its toxic breakdown compounds (hydroxypyridones (DHPs)) was believed to limit potential animal productivity in the absence of intervention. Diets containing greater than 30%, and certainly 100% leucaena, were historically considered incapable of sustaining liveweight gains (LWG) (Jones and Hegarty 1984), without prior inoculation with the rumen bacteria *Synergistes jonesii*. The toxic effects were originally ascribed to the goitrogenic effect of 3,4-DHP depressing T_4 and T_3 levels (Jones *et al.* 1978), however a major pathway of toxicity is the chelation with, and depletion of, essential elements (Tsai and Ling 1971; Stunzi *et al.* 1980).

The observed lack of toxicity in goats consuming moderate (<50%) levels of leucaena, in Bogor, Indonesia (Jones and Lowry 1984) and in Hawaii (Jones and Megarrity 1983) was, at the time, attributed to the natural occurrence of the DHP-degrading ruminal bacterium *S. jonesii* (Jones and Lowry 1984). Similar conclusions were made regarding ruminants in other countries (Indonesia, Vanuatu, Thailand, Malaysia, India, Seychelles, Mauritius and Mexico) with a longstanding history of feeding leucaena (Jones 1994). However, the findings of this thesis indicate that *S. jonesii* is not capable of completely degrading all DHP in ruminants on high leucaena diets. Other relevant findings include: the discovery of the ubiquity of the bacterium and the high propensity for point mutations in the 16S rDNA gene sequence of *S. jonesii* (Padmanabha *et al.* 2014); the dominance of 2,3-DHP as a metabolic endpoint of mimosine degradation (Halliday *et al.* 2014b); and the perpetual presence of high levels of 2,3-DHP in urine of ruminants consuming high leucaena diets without detrimental toxic effects (Halliday *et al.* 2014c).

Accordingly, a new hypothesis was formulated that postulates metabolic conjugation of DHP as the critical pathway in toxicity amelioration.

Despite early evidence supporting this hypothesis (Christie *et al.* 1979), the role of conjugation of DHP in detoxification has not been greatly researched since that time. In fact, the methods employed for measuring DHP intentionally hydrolysed conjugated DHP to the free form, without quantification (Ford *et al.* 1984), and overlooked any protective benefits of the conjugate. In the work of this thesis, despite following the recommended acid-hydrolysis protocols, the more advanced analytical procedures (Chapters 4, 6 and 7) have consistently identified HPLC peaks for what was suspected to be additional remaining conjugated DHP in HPLC chromatograms.

In order to investigate the role of conjugation in reducing toxicity, urine was sampled in Jati Sari hamlet, from bulls fed 100% leucaena which were not only asymptomatic of DHP-toxicity, but were also achieving liveweight gains approaching the genetic potential of *Bos javanicus* bulls (Panjaitan *et al.* 2014) (0.85 kg/day) (Mastika 2003). When bulls from this hamlet were first sampled in 2011 (Chapter 6), they were found to be excreting high levels of DHP, specifically 2,3-DHP. This was consistent with recent findings in which DHP was voided from ruminants primarily in the form of 2,3-DHP. It was apparent that the bulls were able to tolerate high levels of 2,3-DHP without degradation of the toxin.

The first objective of this study was to conduct a longitudinal monitoring survey of urinary DHP excretory patterns in bulls fed 100% leucaena diets, to overcome limitations of the single “spot” samples obtained in previous assessments, and to characterise the isomeric form of DHP excreted. The second objective was to identify and quantify the DHP-conjugate in bulls adapted to high leucaena diets. A refined collection methodology was employed in order to prevent hydrolysis and to preserve the conjugated form of DHP.

If confirmed, the detoxification of DHP via conjugation has the potential to lessen the management requirements of leucaena-fed ruminants worldwide, as reliance upon inoculation with *S. jonesii* to manage leucaena toxicity would no longer be required. This would represent a paradigm shift in the way in which leucaena toxicity is viewed and the issue of toxicity as a barrier to adoption in many countries would be eliminated.

8.2 Materials and methods.

The survey was undertaken in the leucaena-based bull-fattening hamlet of Jati Sari, Rhee village, Sumbawa Island, West Nusa Tenggara (NTB) (8° 25' 10.88" S, 117° 16' 0.46" E) in eastern Indonesia (Plate 8.1). Jati Sari was first identified in the sampling survey of Chapter 6 where bulls were fed diets of up to 100% fresh cut leucaena 3–4 times per day in a cut-and-carry system, while tethered in simple sheds (‘kandangs’).

Two sampling periods were undertaken in this study: the first was a 4-week monitoring period during January 2014; and the second was a 2-day monitoring period in May 2016. The purpose of the second period was to confirm that the process of conjugation was a consistent phenomenon, and to collect rumen fluid with improved methods for preserving RNA.

An improved method for urine sample collection and preservation is also described, which allowed the conjugated form of DHP to be studied.

Plate 8.1: The hamlet of Jati Sari in the foreground, with leucaena planted in rows in the background.

8.2.1 Animal selection and feeding

In the 2014 sampling period, six farmers were selected, from whom 18 Bali bulls (*Bos javanicus*) were monitored. These included 6 existing bulls (average weight 201 ± 11 kg (±s.d.)) which had been fattened on a 100% leucaena diet for >5 months, and 12 bulls (average starting weight 154 ± 37 kg (±s.d.)) which had newly arrived and commenced high-leucaena diets for their first time.

In the 2016 sampling, 10 existing Bali bulls (average starting weight 200 ± 18 kg (±s.d.)) from a single farmer were selected which had been maintained on 100% leucaena diets for 4–6 months.

Animals were fed fresh cut leucaena 4–5 times per day. Leucaena was sourced from trees grown on each individual farmer's land, that were up to 20 years old. Side branches of up to 2 m in length were cut twice per day, and distributed throughout 4–5 feedings. It was common for farmers to collect enough feed to offer bulls a midnight feed to maximise intake and therefore shorten the fattening period. Leucaena was fed *ad lib*, and the time fresh feed was available in front of animals was maximised to encourage increased intake.

These monitoring studies were sanctioned under animal ethics #SAFS/023/15/ACIAR (Appendix 4) and #SAFS/038/12/ACIAR (Appendix 5).

8.2.2 Liveweight measurements

Liveweight measurements for bulls in the first period were already being recorded monthly after overnight fasting from food and water as part of a supplementary longitudinal productivity assessment (Panjaitan *et al.* 2014). Bulls were weighed on arrival and for the duration of their fattening in this hamlet.

8.2.3 Urine sample collection

Urine samples were collected into a plastic receptacle, using a small brush to stimulate the penis sheath to encourage urination. Fresh urine samples were then cleaned through grade 1, 11 µm filter paper. A 9.5 mL subsample was preserved in solution with 0.5 mL 32% hydrochloric acid (HCl), in polypropylene tubes (Dalzell *et al.* 2012). A second subsample was retained for analysis without acid-hydrolysis. To prevent bacterial degradation of DHP, 10 mL of clean urine was passed through a syringe driven filtration system consisting of a GF/C filter and a 0.22 µm Millex Millipore filter, into polypropylene tubes. Samples were then immediately placed on ice and stored at <−20ºC.

In the 2014 period, samples were collected three times per day (at approximately 08:00, 13:00, and 17:00), three days per week (Monday, Wednesday, and Friday), for four consecutive weeks during the month of January 2014.

In the 2016 period, sampling occurred three times at 09:00 and 13:00 on day one, and at 09:00 on day two.

In total, 226 individual urine samples were collected from period 1, and 30 from period 2.

8.2.4 Colorimetric identification and proportion of conjugated DHP

Estimation of the concentration, type of isomer, and proportion of conjugated DHP was conducted colorimetrically based on the iron(III) chloride method of Graham (2010); this was conducted for both the acidified and unacidified urine samples. The immediate colour reaction of unacidified urine was recorded and photographed. The acidic solution of iron(III) chloride facilitated the gradual hydrolysis of the unacidified sample. A corresponding increase in colour intensity of this sample was indicative of hydrolysis of conjugated DHP, which was not able to immediately complex with the iron(III) in solution. The colour reaction of the unacidified sample was monitored through to hydrolysis reaching the same intensity as the acidified urine samples.

The colour intensity of each observation was recorded and assigned a rank from 0–5 adapted from Graham *et al.* (2014). The colour hue of the iron(III) chloride reaction was compared with standards for the following isomers of DHP: 2,3-dihydroxypyridine (2,3-DHP); 2,4-dihydroxypyridine (2,4-DHP); 2,5-dihydroxypyridine (2,5-DHP); 2,6-dihydroxypyridine (2,6-DHP); and 3,4-dihydroxypyridine (3,4-DHP), and the following isomers of mono-hydroxypyridine (HP): 2-hydroxypyridine (2-HP); 3-hydroxypyridine (3-HP); and 4-hydroxypyridine (4-HP) (Plate 8.2).

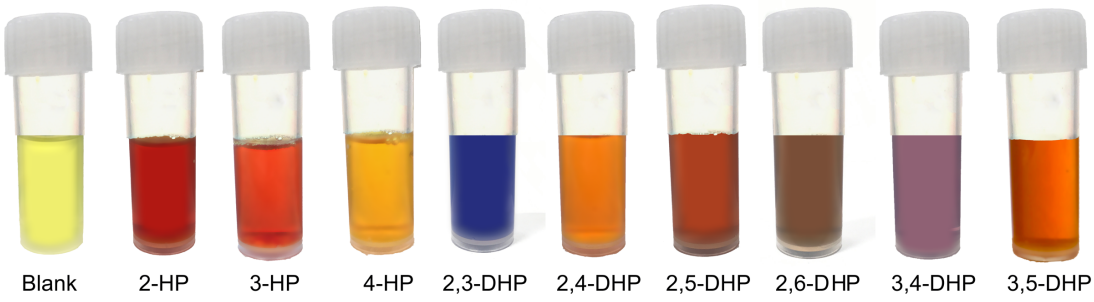


Plate 8.2: Colour development of isomers of hydroxypyridine and dihydroxypyridine compounds with iron(III) chloride solution.

8.2.5 Optimisation of hydrolysis and HPLC confirmation of 2,3-DHP conjugate

The existing method for hydrolysis of DHP to release bound glucuronides reported by Dalzell *et al.* (2012), and adapted from Hegarty *et al.* (1964b), consisted of 1:1 purified urine with 32% HCl. However, this has been shown to be ineffective at achieving complete hydrolysis of strongly bound conjugated DHP, as evidenced by the presence of a related 2,3-DHP peak eluting prior to free 2,3-DHP in chromatograms of urine samples reported in Chapters 4, 5, 6 & 7. The following procedure was conducted to advance the hydrolysis of the conjugate and confirm the peak as conjugated 2,3-DHP.

A representative selection of 10 samples identified by colorimetric assay as having high levels of conjugated 2,3-DHP was chosen. All samples were cleaned with Millex Millipore® 0.45-µm cellulose acetate filters (Sartorius AG, Goettingen, Germany), followed by reverse phase solid phase extraction (RP-SPE) on Maxi-Clean™ 300 mg C18 cartridges (Alltech Associates Inc., Deerfield, IL, USA) to remove particulate and non-polar contaminants. Unacidified urine samples (10 mL) were separated into 2 mL subsamples, and subjected to the following hydrolysis treatments:

1. Minimal acidification (HCl-pH3): A 2 mL subsample was diluted 1:4 with distilled H_2O and the pH adjusted to ≤ 3 with 0.1 M HCl using a Radiometer analytical® automatic titrator.
2. Standard HCl hydrolysis (HCl-WB): A 2 mL subsample was diluted 1:1 with 32% HCl in a sealed polypropylene tube and heated in a water bath at 90ºC for 60 min.
3. Advanced HCl hydrolysis (HCl-HB): A 2 mL subsample was diluted 1:1 with 32% HCl and transferred into a glass vial. The air space was purged with argon prior to sealing with a Teflon sealed cap and placed in a heating block at 105ºC for 60 min.
4. Advanced trifluroacetic acid hydrolysis (TFA-HB): A 2 mL subsample was diluted 1:1 with 4 M trifluroacetic acid (TFA) (Quemener *et al.* 1997) and transferred into a sealed glass vial. The air space was purged with argon prior to sealing with a Teflon sealed cap and placed in a heating block at 110ºC for 35 min.

DHP was quantified with HPLC according to the method of Dalzell *et al.* (2012), with a corrected mobile phase of 25 mM ammonium dihydrogen phosphate, adjusted to pH 2.5, and a Luna 5u C18(2) 150 x 4.6 mm 5 micron Phenomenex column.

Spectral analysis was conducted with an ultraviolet (UV) photodiode array (PDA) detector set to the wavelength range of λ = 190–400 nm. The adsorption spectra of identified peaks based on retention time (RT) were then compared with those of standards of HP and DHP isomers as additional confirmation (Figure 8.1). The integration of peaks for each isomer

was calculated on Shimadzu Lab Solutions SP1 software, version 5.57, at their corresponding wavelength maxima (λ_{max}): λ = 235 nm for 4-HP; λ = 270 nm for 2,4-DHP, 2,5-DHP, 2,6-DHP and 3,4-DHP; λ = 275 nm for 3-HP; λ = 295 nm for 2-HP, 2,3-DHP, and 2,3-DHP conjugate peaks.

The area under the 2,3-DHP and 2,3-DHP conjugate peaks was compared for each sample preparation method to confirm that the conjugated 2,3-DHP peak converted to free 2,3-DHP with increasing hydrolysis.

In order to assess the level of conjugation of DHP, the 226 urine samples from period 1 (2014) collections were analysed using HPLC with minimal acidification (HCl-pH3).

8.2.6 Confirmation of the 2,3-DHP conjugate with HDMS

In addition to HPLC, unacidified filtered urine samples were analysed using high definition mass spectrometry (HDMS) in order to confirm the form of the 2,3-DHP conjugate based on molecular weight of the peaks using time of flight (TOF) MS, and tandem mass spectrometry (MS/MS) on suspected conjugates. Representative samples from 2014 and 2016 samplings (*n* = 11 and *n* = 10, respectively), where an increase in colour was recorded using the colorimetric test after acid hydrolysis, were selected.

As the existing chromatography method was not suitable for introduction to LCMS, the polar hydroxy-pyridinyl compounds were separated by anion exchange resin prior to direct injection to MS, without a column. The anion exchange resin procedure was in substitute for the C18 RP-SPE column and based on the technique presented in Hegarty *et al.* (1979). The exchange resin was prepared by slurry washing 20 g of AG-1 100–200 mesh in chloride form with 30 mL of 50% methanol. The resin was packed into 3 mL tubes to 1 cm below the top with 10 mL of deionised water (DI) using a luer adapter. The resin was then converted to hydroxide form with 15 mL of 1 M sodium hydroxide (NaOH) slowly passed through, followed with 20 mL of DI, ensuring eluate was pH neutral.

Subsamples of urine (4 mL) were adjusted to pH 9 using 4 M NH_4OH dropwise, ensuring that the carboxyl moiety of the conjugate, and the hydroxyl moiety of the free DHP, were fully ionised. After standing for 2 hours, samples were filtered using a 0.45 µm Millex Millipore membrane filter. The resin-packed tubes were cleaned with 2 mL of DI, and 2 mL of air to purge free water from the column. The filtered urine sample (pH ≥9), was slowly (1 drop per second) passed through the column, followed by 2 mL of DI and 2 mL of air, and collected in a 15 mL centrifuge tube.

The bound DHP was slowly (1 drop per second) eluted using 10 mL of 5 M formic acid in 50% methanol, followed by 2 mL of air and eluate collected in a 50 mL centrifuge tube. Tubes were stored at <5 °C prior to centrifugal evaporation with a miVac Quattro concentrator to remove the formic acid and methanol. The 15 mL tubes were spun at 50ºC for 3 hours using H_2O method; 50 mL tubes were spun at 40ºC for 4 hours using the –OH method, to reduce the volume to <4 mL. Millipore water was used to reconstitute samples to the original volume of 4 mL.

For each sample, two additional 2 mL subsamples were prepared using the minimal acidification (HCl-pH3) and the standard HCl hydrolysis (HCl-WB) procedures previously described.

5µL of the eluate from the anion exchange resin was injected into a Waters Acquity UPLC system without an LC column, using an isocratic mobile phase of 50:50 water/acetonitrile with 0.1% formic acid, at a flow rate of 0.3 mL/min. The flow from the system was connected to a Waters Synapt G2-S High Definition Mass Spectrometer. A mass spectrum was collected in the range 50–400 Da every 0.5 sec, for a 1 minute period. Data were collected in both positive and negative mode.

Tandem mass spectrometry (MS/MS) with collision-induced dissociation (CID) using a collision energy ramp of 10–30 V was performed for peaks of interest. The major fragment peaks were then matched to the masses corresponding to the breakdown projects of the conjugates. Data were analysed using MassLynx software. All samples analysed by MS were also analysed with HPLC as described above.

8.2.7 Rumen fluid collection

Rumen fluid samples were collected twice during sampling in 2014 (commencement and conclusion of monitoring) and once during the 2016 sampling from each of the bulls. The method of collection as described in Graham *et al.* (2013) was updated with the use of a catheter-tip 50 mL syringe in place of a vacuum pump to more efficiently collect samples and minimise animal stress. A minimum of 100 mL of rumen fluid was extracted and strained through 4 layers of muslin cloth into a collection container. Duplicate 8 mL sub-samples were withdrawn from the centre of the collected fluid, and added to 11.5 mL of 100% ethanol for 2014 collections; and 8 mL of RNALater™ Stabilisation Solution (Thermofisher Scientific) for 2016 collections, in 50mL falcon tubes. Tubes were inverted for 10 seconds and stored immediately on ice. After 48 hours at ~2ºC, samples were centrifuged at 6000 × g for 15 minutes and the supernatant discarded. The pellet was re-

suspended in 20 mL of 70% ethanol and exported to Australia at ambient temperature for PCR isolation.

DNA analysis for the presence of *S. jonesii* was conducted following the method described in Chapter 5, and rRNA based reverse transcriptase quantitative PCR (qPCR) was conducted using the method briefly described below.

8.2.8 Extraction of DNA and RNA and detection of S. jonesii

After DNA extraction with a Sodium dodecyl sulphate (SDS) lysis buffer, RNA was extracted using the RNeasy® Mini Kit (cat. No. 74104, QIAGEN) protocol with the following modifications: 500 µL RLT buffer was added to the aqueous phase, mixed well, and 500 µL ethanol was added to precipitate RNA.

The 3 primer pairs described in Chapter 5, plus an additional primer: Sj449R (5'-CGT CAC TCG CTT CTT CCC GC-3'), were used to target *S. jonesii*. PCR products were sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit and analysed on an ABI3130xl automatic sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were aligned and analysed using AlignX software (Vector NTI software, Invitrogen) against 16S sequences from *S. jonesii* 78.1 as the reference.

The gDNA from rumen digesta samples was diluted approximately to 50–100 ng/µL and used in a 25 µL PCR. Nested PCR (Chapter 5) increased the sensitivity of *S. jonesii* detection, with positive products confirmed by cloning and sequencing. Multiple alignment of all sequences was done using Vector NTI's Advance 11 software (Invitrogen, Life Technologies, USA) AlignX module using Clustal W with *S. jonesii* ATCC 49833 (78-1) type strain 16S rDNA as the reference.

8.2.9 Statistical analyses

Data were analysed using Microsoft Excel computer software (©2016, Microsoft, Redmond, WA, USA) and Minitab® statistical software 16 (©2010, Minitab Inc., State College, PA, USA). One-way ANOVA tests were used to analyse total D/HP and proportion of conjugated 2,3-DHP with respect to the number of weeks since leucaena-feeding commenced, time of sampling and individual animal. Means were compared using Fisher's least significant difference (LSD) test ($P < 0.05$). Descriptive statistics were used to summarise mean values with standard errors listed. A probability level of $P < 0.05$ was considered statistically significant, while a *P*-value between 0.05–0.10 was considered a moderate trend.

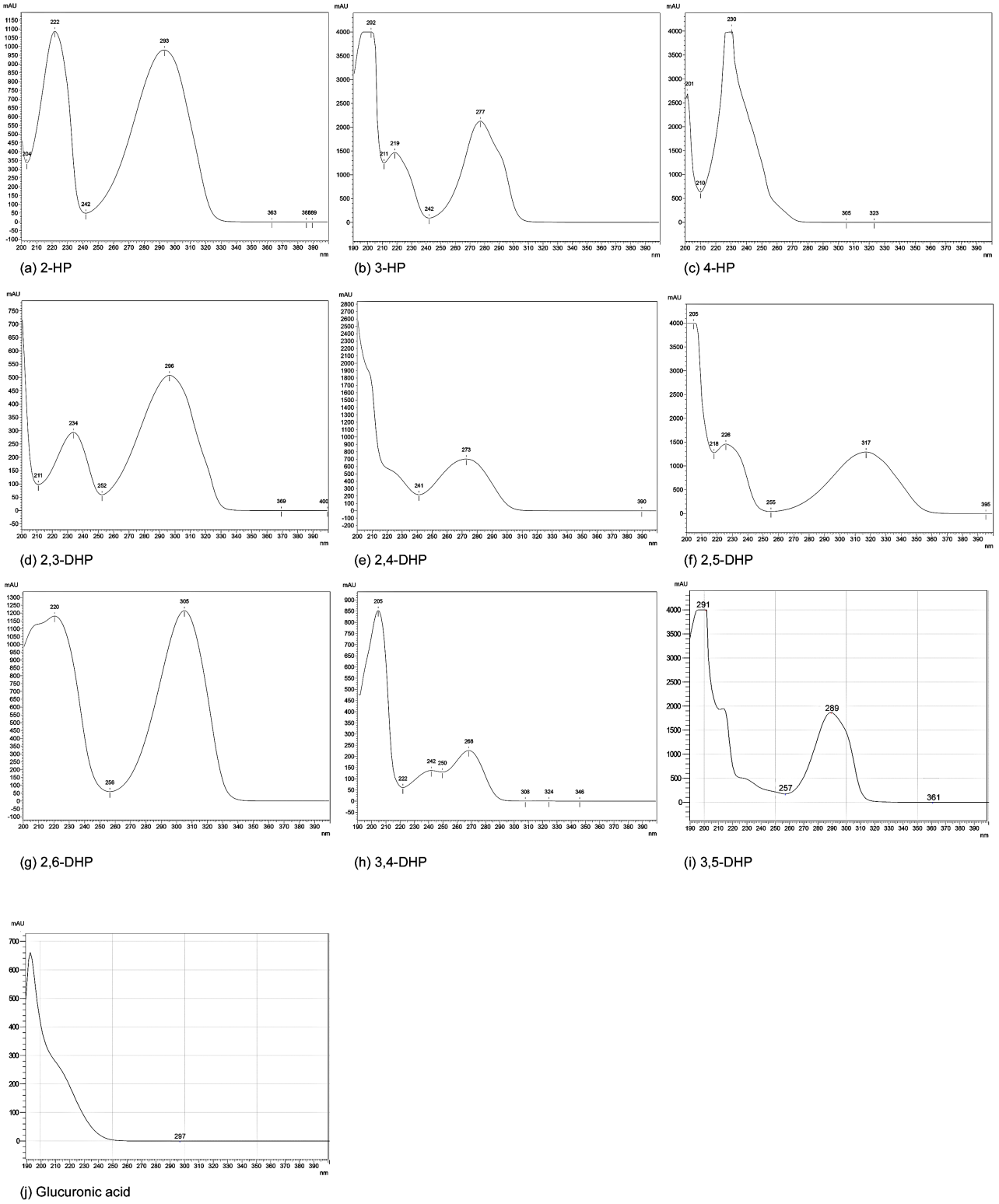


Figure 8.1: UV absorption spectra of: (a) 2-hydroxypyridine (2-HP); (b) 3-hydroxypyridine (3-HP); (c) 4-hydroxypyridine (4-HP); (d) 2,3-dihydroxypyridine (2,3-DHP); (e) 2,4-dihydroxypyridine (2,4-DHP); (f) 2,5-dihydroxypyridine (2,5-DHP); (g) 2,6-dihydroxypyridine (2,6-DHP); (h) 3,4-dihydroxypyridine (3,4-DHP), (i) 3,5-dihydroxypyridine (3,5-DHP); and (j) glucuronic acid.

8.3 Results

8.3.1 Bull LWG and feed chemical composition

The mean fattening period was 151±15 days, with a range of 28–247 days. The average LWG for bulls fattened during 2014 samplings was 0.51±0.02 kg/d with the greatest individual LWG of 0.71 kg/d, over a 152-day period.

The crude protein content of the leucaena feed offered was consistent with previous values (Chapter 7) obtained for fresh-cut leucaena leaf material. Bulls were able to selectively strip and eat fresh leaf material from the branches offered via prehension with their tongue and teeth. Small stem material was also consumed up to a diameter of ~10 mm. The feed quality of leaf plus small stem was similar to leaf only material (Table 8.1). The fibre content and mineral composition were also consistent with results obtained in Chapter 7.

Table 8.1: Chemical composition (g/kg DM) of fresh leucaena leaf and leucaena leaf and stem offered to *Bos javanicus* bulls.
CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre

Parameter	Leucaena leaf	Leucaena leaf and stem
CP (g/kg DM)	239 ± 5	203 ± 39
NDF (g/kg DM)	420 ± 9	397 ± 43
ADF (total) (g/kg DM)	343 ± 6	308 ± 36
Fe (mg/kg)	109 ± 20	78 ± 8
Zn (mg/kg)	12 ± 1	13 ± 2

8.3.2 HPLC DHP results of the longitudinal survey

The results of the 2014 HPLC analysis of samples collected during the 4 weeks of period 1 (n = 226), with minimal acidification (HCl-pH3), indicated that the conjugate forms of DHP were preserved with almost all (97 ± 1%) mimosine-derived pyridinyl metabolites (HP and/or DHP isomers—D/HP) excreted in conjugate form (Figure 8.2). Of note was the finding that animals sampled during their first week consuming 100% leucaena diets were conjugating DHP at this level with no detected lag period for the up-regulation of conjugation to occur.

The 2,3-DHP isomer (in the primary conjugated form) represented the majority of D/HP metabolites (97 ± 1%) (Figure 8.2), with all possible isomers screened (Table 8.2). The isomers of 2-HP, 2,4-HP, 2,5-DHP, 2,6-DHP, and 3,5-DHP were never detected in HPLC, while 3-HP, 4-HP and 3,4-DHP were detected only sporadically and at low concentrations.

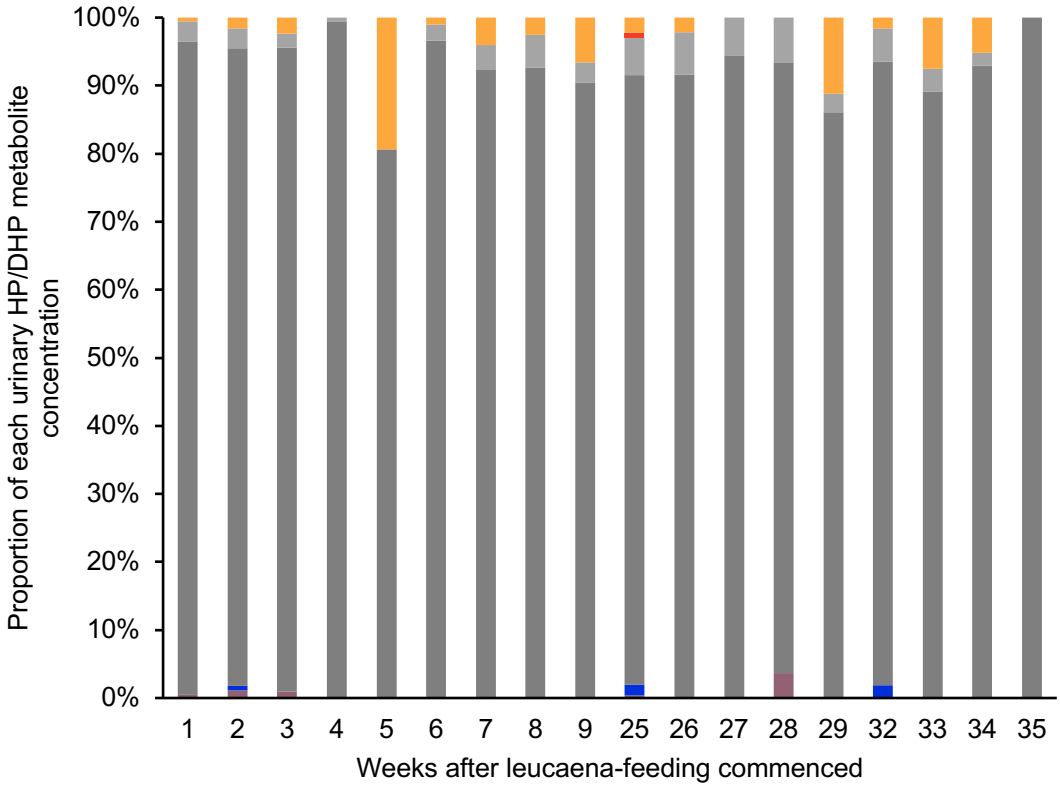


Figure 8.2 Relative percentages of metabolite excretion in urine of *Bos javanicus* bulls sampled during a 1-month period in 2014 by number of weeks since leucaena-feeding first commenced (values are means of all samples collected in that week from corresponding animals).

3,4-DHP, **violet**; 2,3-DHP, **blue**; primary 2,3-DHP conjugate (RT = 5.3 m), **dark grey**; secondary 2,3-DHP conjugate (RT = 3.7 m), **light grey**; 3-HP, **red**; and 4-HP, **orange**.

There was no significant difference in the proportion of conjugated 2,3-DHP excreted regardless of the number of weeks animals had been consuming leucaena (P = 0.98) 1-way ANOVA or the time of day samples were collected (P = 0.20) 1-way ANOVA, however there was a difference among animals (P = 0.00) 1-way ANOVA (Table 8.3); animals 6–10 & 12 were new to 100% leucaena diets. Instances where the proportion of conjugated 2,3-DHP was 0% corresponded to samples where the only metabolite was 4-HP, present at low (~100 mg/L) levels, with one instance of 3,4-DHP. This occurred in 8 samples for animals that had been consuming leucaena for 2–26 weeks.

The mean amount of total pyridinyl metabolites (HP+DHP) excreted was variable between weeks since leucaena-feeding commenced (P = 0.01) 1-way ANOVA (Figure 8.3) and time of day the samples were collected (P = 0.00) (Figure 8.4); samples collected at 08:00 had the lowest mean total D/HP concentrations (733±108 mg/head) whereas samples collected at 13:00 had the highest mean total D/HP concentrations (1418±168 mg/head). Of all collections, a total of 29 samples had <100 mg/L D/HP present, which if assessed in isolation as “spot” samples would wrongly provide a false negative result.

Table 8.2: Chemical structure of all mono- and di-hydroxypyridine compounds with their UV absorption maxima and HPLC retention time.

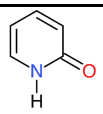
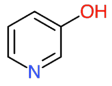
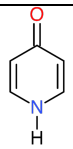
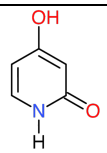
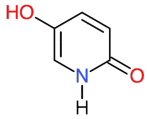
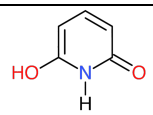
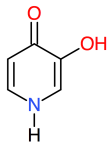
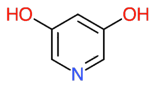
Compound name/s	Common abbreviations	Structure	Molecular formula	Molecular weight (g/mol)	UV λ_{max} (nm)	HPLC retention time (m)
2-hydroxypyridine; 2-(1*H*)-pyridone	2-HP; 2-Po		C_5H_5NO	95.10	195, 221, 293	7.21
3-hydroxypyridine	3-HP		C_5H_5NO	95.10	200, 277, 219	2.27
4-hydroxypyridine; 4-(1*H*)-pyridone	4-HP; 4-Po		C_5H_5NO	95.10	229, 201	2.41
2,3-dihydroxypyridine; 3-hydroxy-2(1*H*)-pyridone	2,3-DHP; 3,2-HPo		$C_5H_5NO_2$	111.1	197, 296, 234	6.51
2,4-dihydroxypyridine; 4-hydroxy-2(1*H*)-pyridone	2,4-DHP; 4,2-HPo		$C_5H_5NO_2$	111.1	200, 272	4.89
2,5-dihydroxypyridine; 5-hydroxy-2(1*H*)-pyridone; 3,6-dihydroxypyridine; 3-hydroxy-6(1*H*)-pyridone	2,5-DHP; 2,5-HPo; 3,6-DHP; 3,6-HPo		$C_5H_5NO_2$	111.1	210, 224, 318	3.68
2,6-dihydroxypyridine; 6-hydroxy-2(1*H*)-pyridone	2,6-DHP; 6,2-HPo		$C_5H_5NO_2$	111.1	305, 220	5.27
3,4-dihydroxypyridine; 3-hydroxy-4(1*H*)-pyridone	3,4-DHP; 3,4-HPo		$C_5H_5NO_2$	111.1	205, 268, 242	2.46
3,5-dihydroxypyridine	3,5-DHP		$C_5H_5NO_2$	111.1	201, 289	2.65

Table 8.3: Mean proportion of conjugated 2,3-DHP of total D/HP metabolites by animal.

Means with different subscripts are significantly different at $P < 0.05$

	Proportion of (primary) conjugated 2,3-DHP (%)		
Animal no	Mean±s.e.	Range	*n*
1	$100\pm0_{a}$	100–100	18
2	100_{abcdef}	100–100	1
3	$98\pm1_{ad}$	91–100	15
4	$98\pm1_{ad}$	91–100	16
5	$92\pm6_{ad}$	0–100	16
6	$86\pm8_{def}$	0–100	12
7	$87\pm4_{abcdef}$	75–100	7
8	$95\pm2_{ad}$	81–100	13
9	$86\pm1_{abcdef}$	80–89	12
10	$87\pm4_{abcdef}$	59–100	9
11	$75\pm8_{cf}$	0–100	21
12	$95\pm2_{ad}$	89–100	11
13	$91\pm8_{ad}$	0–100	13
14	$73\pm10_{bcef}$	0–100	12
15	$99\pm1_{ad}$	91–100	12
16	$99\pm1_{ad}$	92–100	12
17	$92\pm1_{abcdef}$	90–93	5

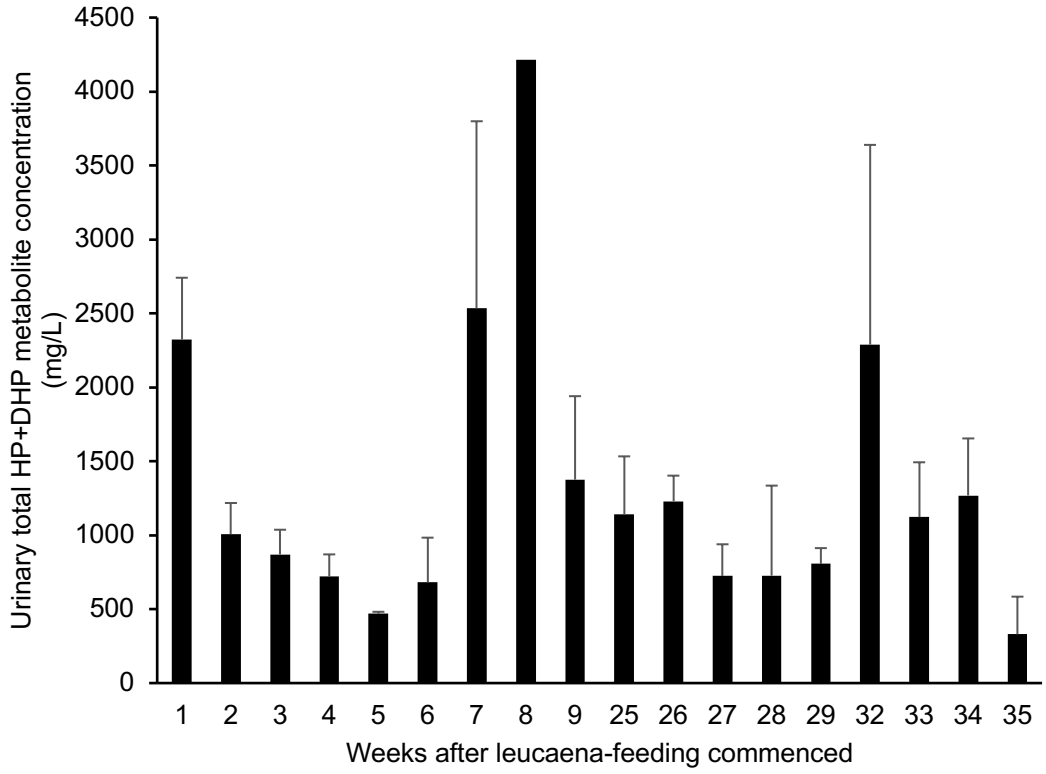


Figure 8.3: Mean (+s.e.) total HP+DHP excretions of *Bos javanicus* bulls sampled during a 1-month period in 2014 by number of weeks since leucaena-feeding first commenced (values are means of all samples collected in that week from corresponding animals).

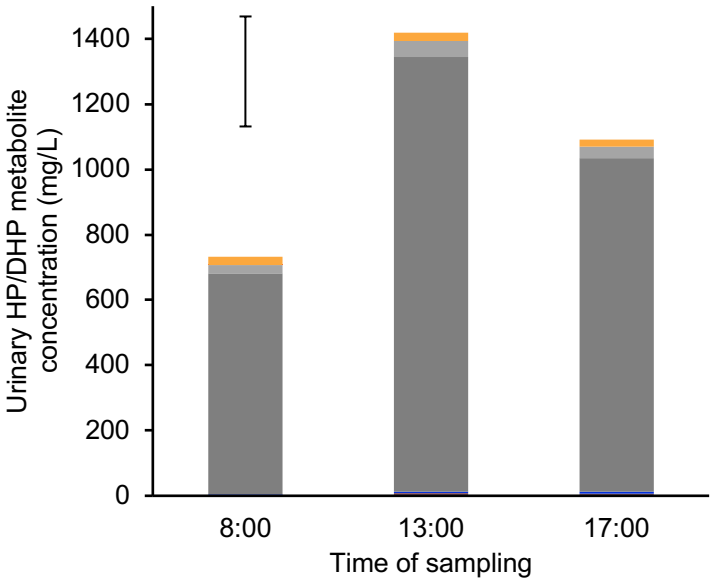


Figure 8.4: Mean excretion of metabolites at the 3 sampling times collected from *Bos javanicus* bulls during a 1 month period in 2014. Bar indicates 1 x $LSD_{0.05}$ where the mean total D/HP excreted was significant ($P < 0.05$).

3,4-DHP, **violet**; 2,3-DHP, **blue**; primary 2,3-DHP conjugate (RT = 5.3 m), **dark grey**; secondary 2,3-DHP conjugate (RT = 3.7 m), **light grey**; 3-HP, **red**; and 4-HP, **orange**.

8.3.3 Quantification of D/HP conjugates

While virtually no free 2,3-DHP (RT = 6.5 m) was detected in samples prepared with minimal acidification (HCl-pH3) (Figure 8.5 & Figure 8.6; **green trace**), a conjugated 2,3-DHP peak was detected at a RT of 5.3 m, more than 1 m prior to the 2,3-DHP peak. This primary 2,3-DHP conjugate had a similar (98% similarity) UV absorption spectra as 2,3-DHP (Figure 8.7), and was identical to the 2,3-DHP conjugate identified in previous chapters. The increased polarity of conjugated DHP resulted in a reduced RT, eluting sooner from the RP-C18 HPLC column.

This peak was confirmed as conjugated 2,3-DHP by sequential hydrolysis of the sample, as the standard HCl hydrolysis procedures (HCl-WB) (Figure 8.5; **red trace**) resulted in a shift in area of the primary 2,3-DHP conjugate to free 2,3-DHP. Despite following the method of the published best practices an average of 77 ± 5% and 61 ± 2% of 2,3-DHP, collected in 2014 and 2016, respectively, remained in conjugated form. Quantification of free 2,3-DHP following this procedure, integrating the area of the curve at the RT of the 2,3-DHP standard, greatly underestimated the amount of 2,3-DHP in the urine.

Near complete hydrolysis of conjugated DHP was achieved following the advanced HCl (HCl-HB) (Figure 8.5; **blue trace**) methods. The same trend was observed in samples collected in 2016 (Figure 8.6). The advanced trifluroacetic acid (TFA-HB) method achieved similar hydrolysis as HCl-HB (Figure 8.8).

In screening for all mimosine-derived pyridinyl urine metabolites, the mono-hydroxypyridines 3-HP and 4-HP were detected, with 3-HP being greater in abundance (Figure 8.5). These were commonly conjugated, as acid hydrolysis increased their concentration at the RT of the corresponding standard (Figure 8.8).

A secondary 2,3-DHP conjugate was also identified with a greatly reduced retention time (3.7 m) (Figure 8.5). UV spectral analysis of this peak (Figure 8.7) confirmed a 95% similarity to the free 2,3-DHP standard, and 98% similarity to the primary 2,3-DHP conjugate (RT = 5.3 m). This peak also reduced after acid hydrolysis. While not conclusive, it is speculated that this may be a sulphate conjugate of DHP (Pang *et al.* 1994).

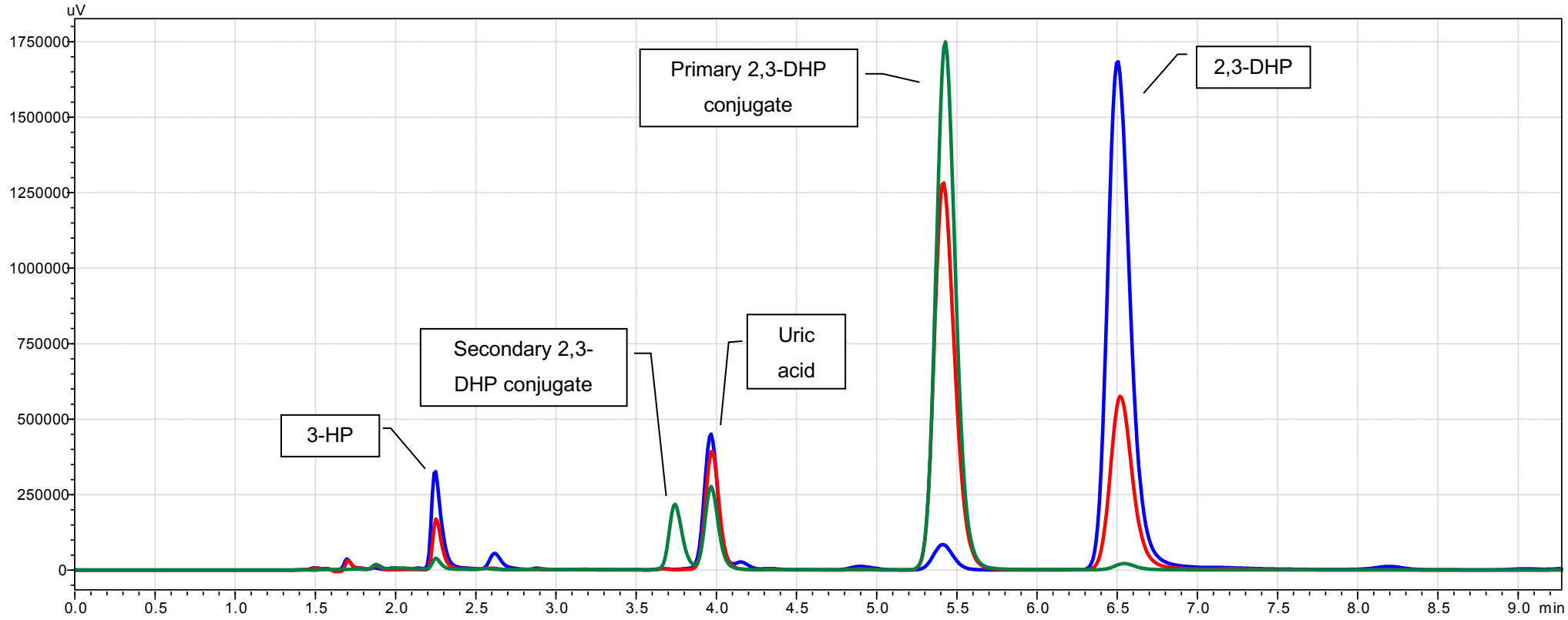


Figure 8.5: Overlaid chromatograms at λ = 295 nm of a single urine collection from a representative animal (99) from 2014 sampling with the following methods: HCl-pH3, **green**; HCl-WB, **red**; and HCl-HB, **blue**.

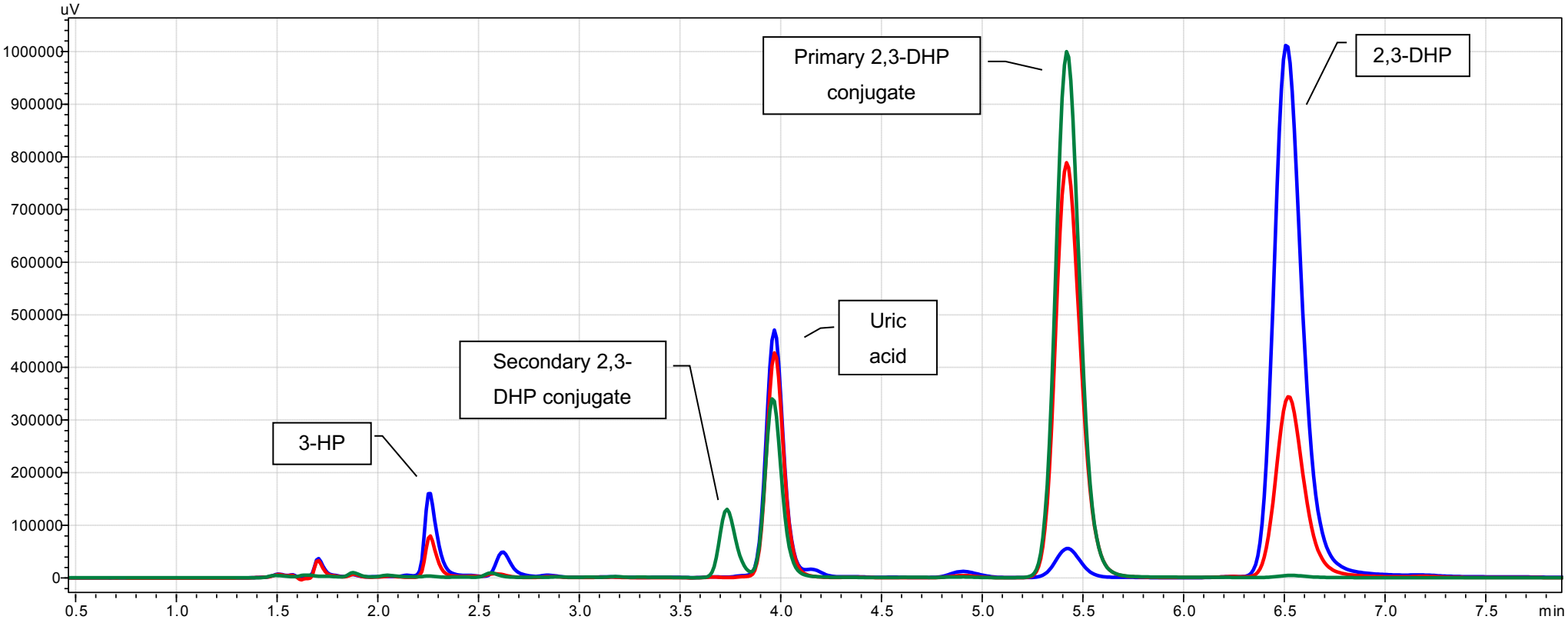


Figure 8.6: Overlaid chromatograms at λ = 295 nm of a single urine collection from a representative animal (1609) from 2016 sampling with the following methods: HCl-pH3, **green**; HCl-WB, **red**; and HCl-HB, **blue**.

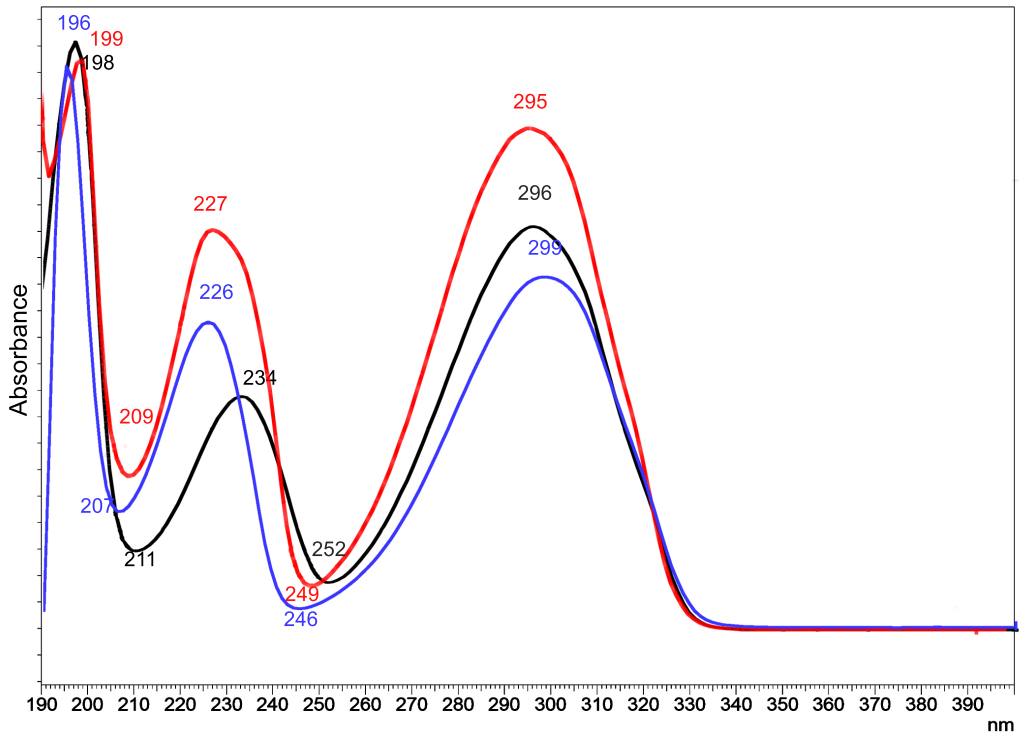


Figure 8.7: Overlaid UV absorption spectra for: 2,3-DHP standard, **black**; primary 2,3-DHP conjugate at RT = 5.3 m, **red**; and secondary 2,3-DHP conjugate at RT = 3.7 m, **blue**.

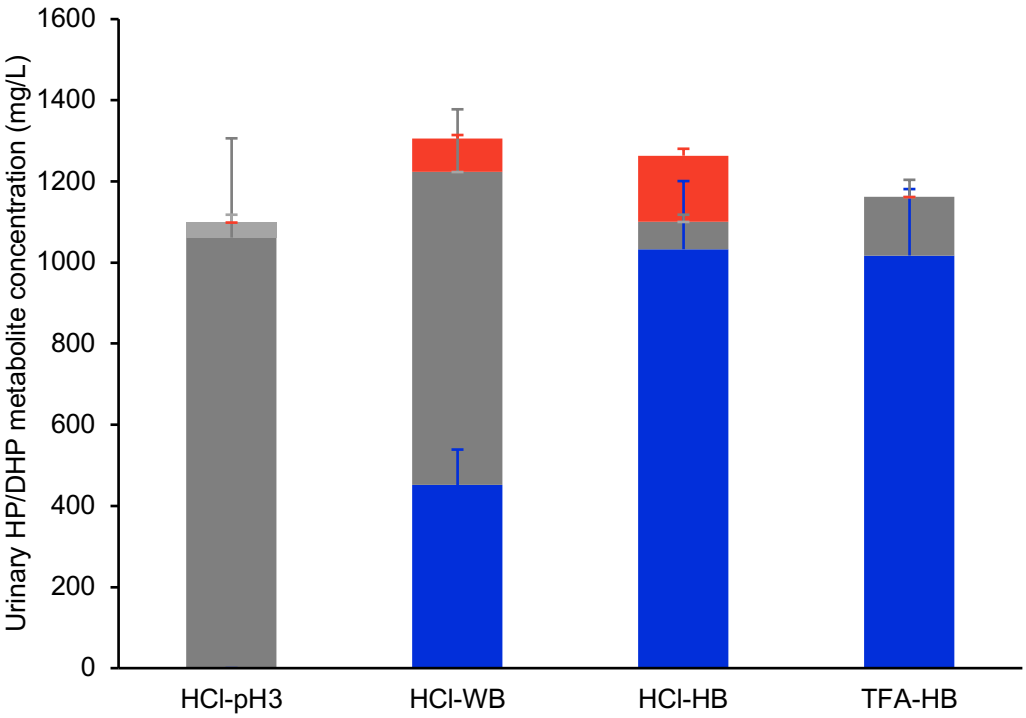


Figure 8.8: Mean (+s.e.) values of metabolites present in 2016 samplings based on the following methods: HCl-pH3; HCl-WB; HCl-HB; TFA-HB.

2,3-DHP, **blue**; primary 2,3-DHP conjugate, **dark grey**; secondary 2,3-DHP conjugate, **light grey**; 3-HP, **red**; and 4-HP, **orange**; present in 2016 samplings based on the following methods: HCl-pH3; HCl-WB; HCl-HB; TFA-HB.

8.3.4 Identification of D/HP conjugates

The use of HDMS was employed to identify the form of conjugate for both DHP and HP. Time of flight (TOF) identification was conducted in both electrospray negative and positive, to account for the different ionisation modes of the free and conjugated forms of D/HP. The common conjugate is that with glucuronic acid (Hegarty *et al.* 1979), combining in a condensation reaction to produce a DHP-3-*O*-glucuronide (DHP-*O*-G) conjugate (Figure 8.9) at a mass of ~287.23 g/mol, and a HP-3-*O*-glucuronide (HP-*O*-G) conjugate at a mass of 271.23 g/mol.

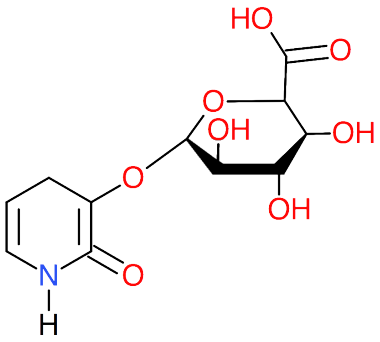


Figure 8.9: 2,3-DHP-3-*O*-glucuronide conjugate, adapted from Galanello (2007).

The analysis of extracted anionic compounds identified both a corresponding DHP conjugate species at the mass −1 charge ($m-H^-$) of 286.13 g/mol and a HP conjugate of $m-H^-$ of 270.09 g/mol in electrospray negative (ESI−) (Figure 8.10a). These conjugates were also detected in electrospray positive (ESI+), with the mass +1 charge ($m+H^+$) of 288.09 and 272.09 g/mol, respectively (Figure 8.10b), although at a reduced intensity. The free forms of DHP and HP were also detected in electrospray positive, with a $m+H^+$ of 112.05 and 96.05 g/mol, respectively. The initial anion resin eluate (“cations”) was negative for any D/HP compounds, free or conjugated.

Tandem MS/MS was performed on the conjugated species of DHP and HP to confirm the glucuronide conjugate. Detected in ESI− MS/MS for the HP-*O*-G conjugate (270.13 g/mol) (Figure 8.11a) was the original HP-*O*-G conjugate, as well as free HP (94.05 g/mol) and the corresponding fragmented glucuronic acid [minus the molecular weight of H_2O, due to the condensation reaction undertaken in the initial conjugation (G.A. – H_2O)] (175.07 g/mol). A proportion of the conjugated form remained intact due to the ramped voltage applied to induce disassociation, and the large number of unidentified peaks was due to the lack of chromatography prior to injection.

Tandem MS/MS of the DHP-*O*-G conjugate in ESI− (Figure 8.11b) similarly resulted in the detection of DHP-*O*-G (286.13 g/mol), free DHP (110.06 g/mol) and G.A. – H_2O (175.08 g/mol).

Although the use of HDMS was not quantitative, all samples from 2014 and 2016 collections were positive for both free and conjugated DHP and HP via TOF and MS/MS analysis. The quantification of these compounds performed using HPLC (Figure 8.12) confirmed that the majority of D/HP was maintained in conjugated form in the “anion” solution.

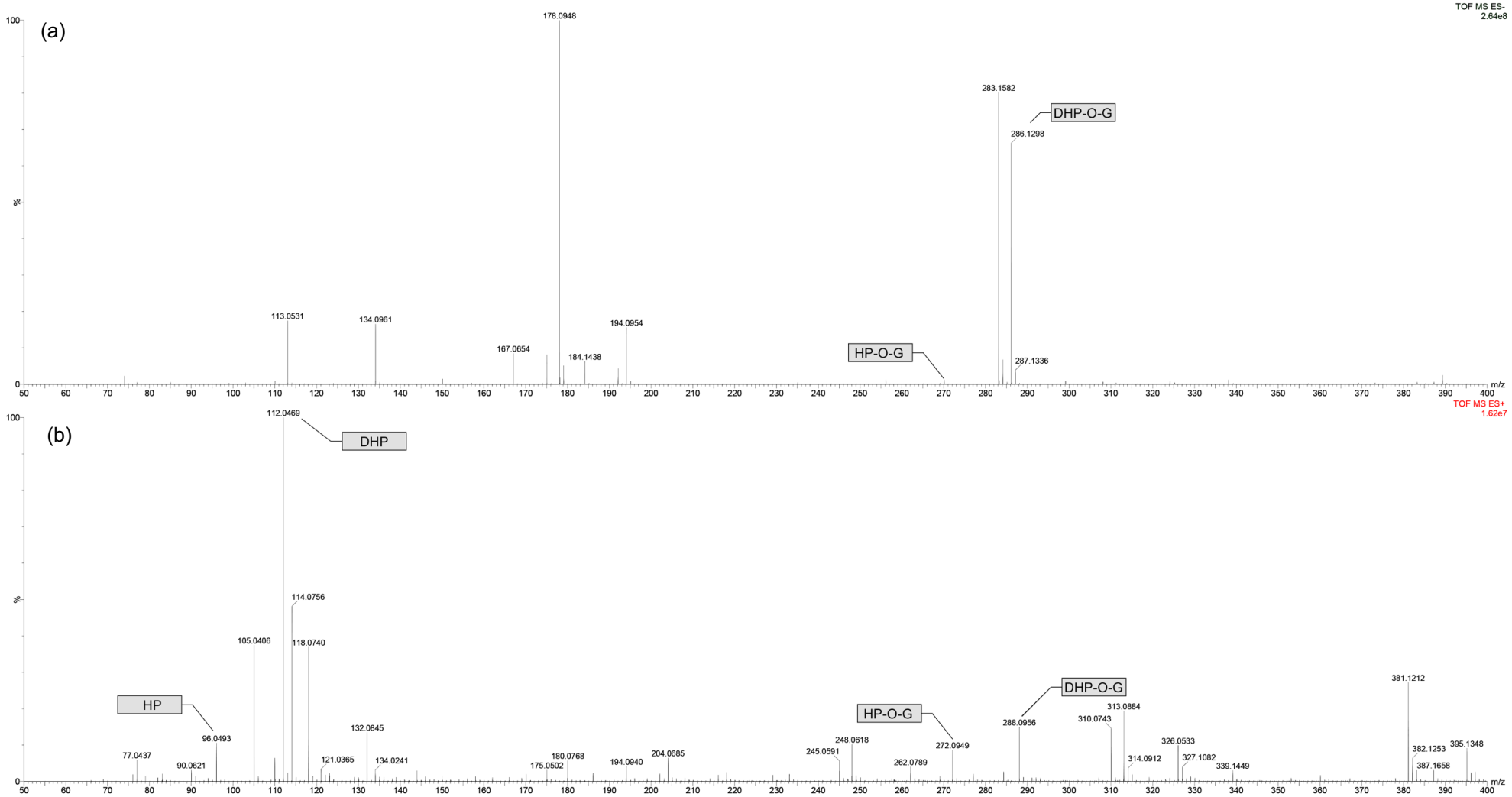


Figure 8.10: Molecular identification based on time of flight (TOF) MS from sample 383C from the 2014 collection in: (a) electrospray negative; and (b) electrospray positive.

HP: hydroxypyridine/pyridone; DHP: dihydroxypyridine/hydroxypyridone; HP-O-G: HP-*O*-glucuronide; DHP-O-G: DHP-*O*-glucuronide

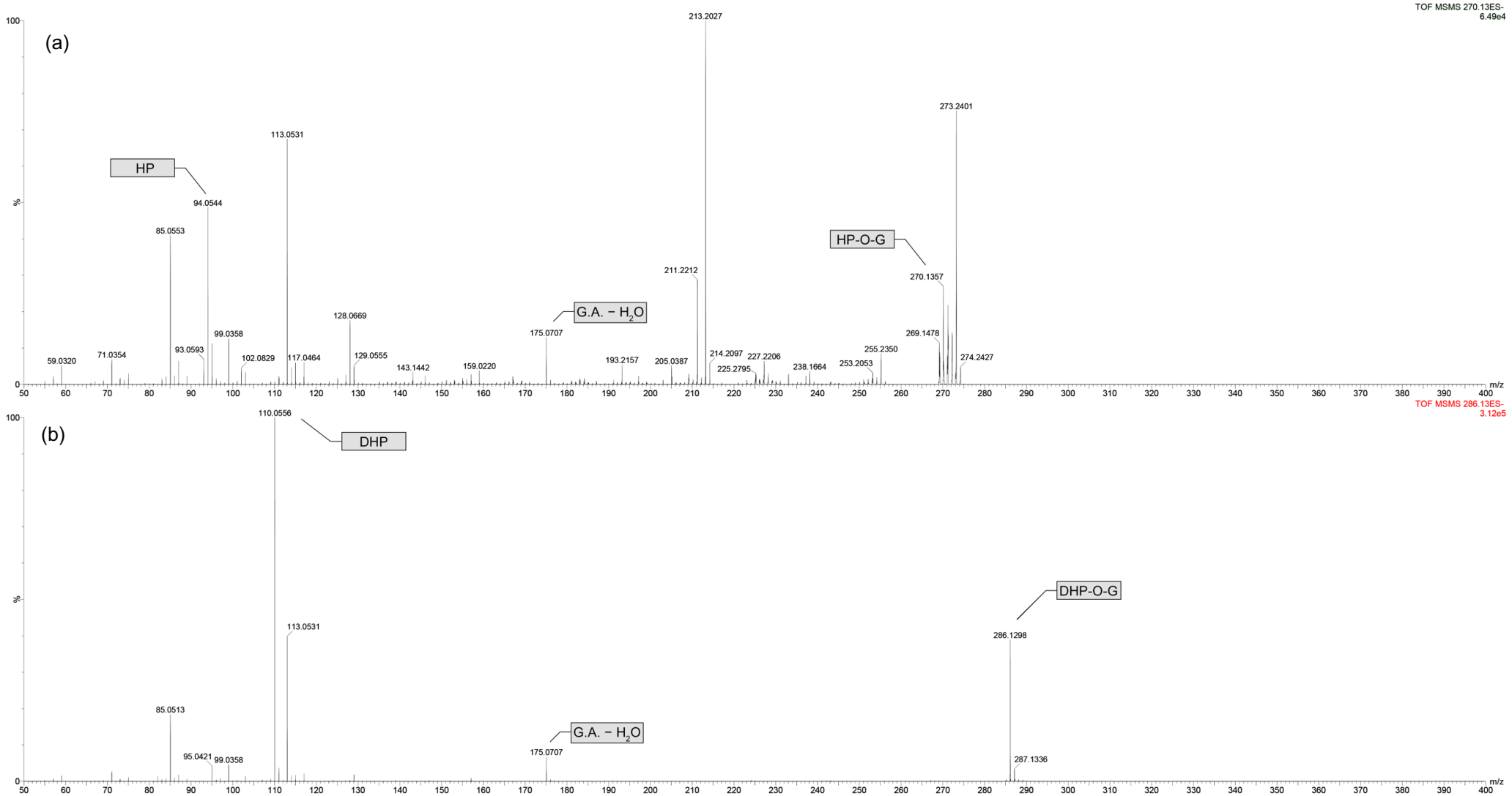


Figure 8.11: Molecular identification of tandem MS/MS results of sample 383C from the 2014 collection in electrospray negative with CID at: (a) m/z 270 for the HP-*O*-G conjugate; and (b) m/z 286 for the DHP-*O*-G conjugate.

HP: hydroxypyridine/pyridone; DHP: dihydroxypyridine/hydroxypyridone; HP-O-G: HP-*O*-glucuronide; DHP-O-G: DHP-*O*-glucuronide; G.A. − H_2O: glucuronic acid – molecular weight of water

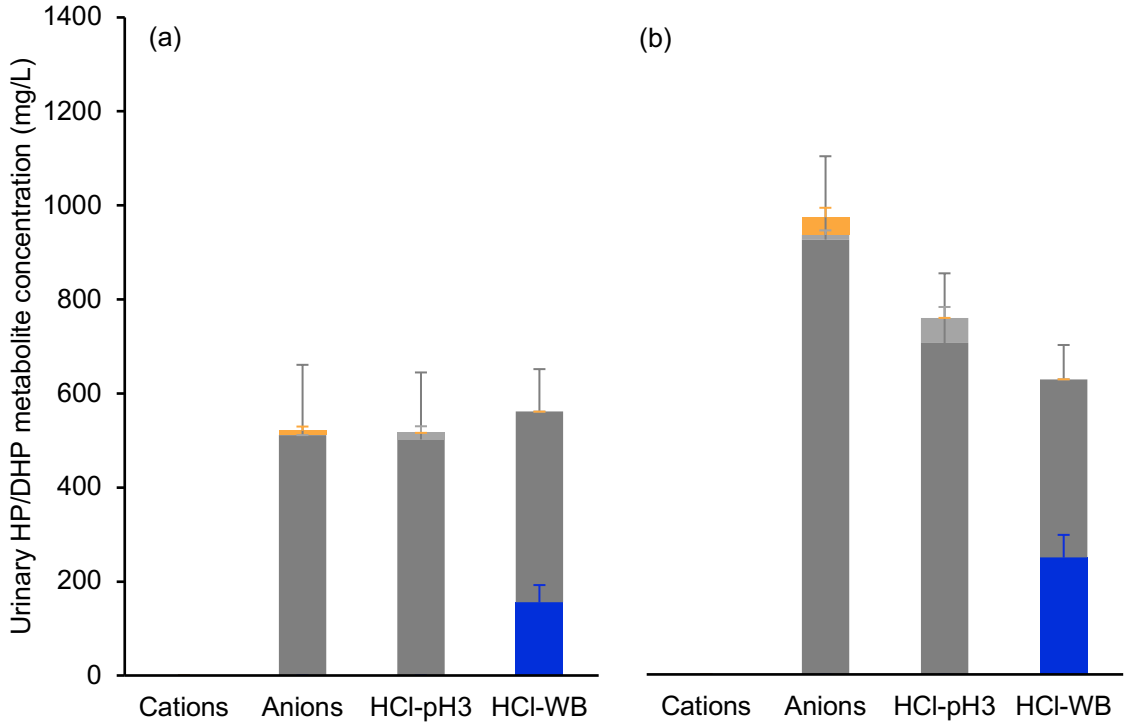


Figure 8.12: Mean values of the metabolites present from: (a) 2014; and (b) 2016 samplings in representative samples chosen for HDMS prepared with the following methods: initial eluate from anion exchange resin, cations; eluted anions from anion exchange resin, anions; minimal acidification, HCl-pH3; partially hydrolysed DHP, HCl-WB.
2,3-DHP, **blue**; primary 2,3-DHPconjugate, **dark grey**; secondary 2,3-DHP conjugate, **light grey**; and 4-HP, **orange**.

8.3.5 Colorimetric results

In the past, iron(III) chloride colorimetric tests have been used extensively to confirm the approximate amount of DHP in urine (Graham *et al.* 2014). The results of this study have identified that the validity of the test depends completely on the effectiveness of the hydrolysis method. For this reason, colorimetric tests were utilised in the selection of conjugated samples for further analysis using HDMS. An initial lack of blue colour (Plate 8.3a), followed by dark blue/black colour development after acid hydrolysis (Plate 8.3b) indicated high levels of conjugated 2,3-DHP. The assigned rank of the colorimetric tests was moderately correlated with the corresponding HPLC results, with an exponential curve ($y = 37.20e^{0.73x}$; $R^2 = 0.85$) (Figure 8.13). The estimation of the percentage of DHP conjugated was also related. Thus, interpretation of on-site colorimetric tests has the capacity to provide an approximate assessment of the range of D/HP isomers present and the level of conjugation in urine.

A shortcoming in the method of hydrolysis was that it was only 23–40% effective, thus underestimating the total amount of DHP present. However, the detection range for the colorimetric method for 2,3-DHP was limited to a maximum of ~2000 mg/L, above which

any additional D/HP was masked by the resultant intense dark blue/black colour. The initial pink/red colour in the sample tested in Plate 8.3a corresponded to 197 mg/L 3-HP, however the method requires further work to advance the test to scale. Further, several isomers of D/HP produced a similar colour, and when multiple isomers were present the colour mixture was not able to be easily interpreted with the naked eye. Despite these limitations, the test was effective in estimating the level of conjugation, and provided a more accurate assessment of the toxicity status of the animal.

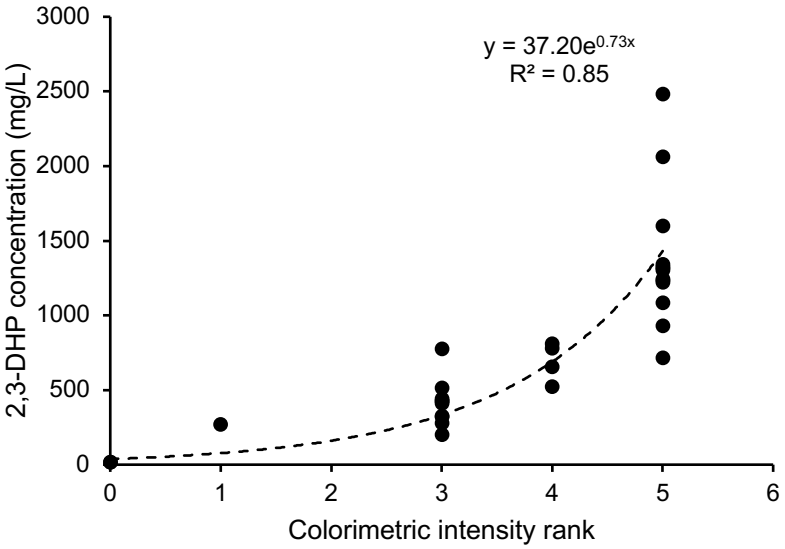


Figure 8.13: Calibration curve of 2,3-DHP estimation based on colorimetric rank against HPLC results after normal acid hydrolysis procedures HCl-WB.

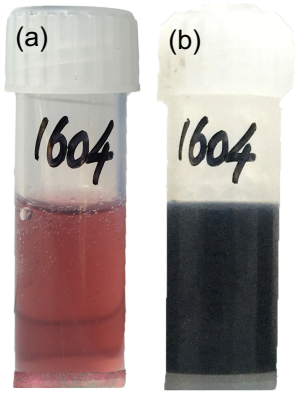


Plate 8.3: Initial colorimetric test result of: (a) filtered unacidified urine; and (b) the same sample after acid-hydrolysis (HCl-WB). The concentration of 2,3-DHP-*O*-G in (a) was 1300 mg/L; of which 65% remained conjugated after standard acid hydrolysis procedures (HCl-WB) (b). This sample contained 197 mg/L 3-HP after complete hydrolysis (HCl-HB).

8.3.6 PCR detection of S. jonesii

Rumen fluid from 17 animals, preserved in 70% ethanol, sampled in 2014 was tested with all samples positive for *S. jonesii* at their final collection based on qPCR RNA. However, only 82% of samples (*n* = 14) were positive for detection of *S. jonesii* at the initial collection. Two of the three animals that were negative at their first collection were new to high leucaena diets and were sampled during their first week on leucaena. The detection levels of *S. jonesii* for gDNA-based nested PCR was much lower with only 1 bull positive at the first sampling (3% of all collections).

When samples were collected in 2016 (*n* = 10) and preserved in RNAlater®, 100% of samples analysed based on qPCR RNA were positive for *S. jonesii*; none of these animals were positive when analysed with gDNA-based nested PCR.

The RNA analysis method had a 1000-fold increase in sensitivity of detection compared with DNA analysis. The finding that samples which tested negative for *S. jonesii* based on DNA analysis were positive based on RNA analysis confirmed that the populations of *S. jonesii* were at the limits of detection for nested DNA PCR (Chapter 5). Despite its increased sensitivity, RNA based PCR is not suitable for quantification of *S. jonesii* as it is based on an unknown number of sequence copies that may not represent individual bacteria.

8.4 Discussion

The results of this study point to conjugation as the major protective mechanism against leucaena toxicity for Bali bulls and validate the findings of previous "spot" samplings throughout Indonesia and Australia, where high urinary 2,3-DHP concentrations were recorded, largely conjugated, and in animals that were asymptomatic. These findings confirm that 100% leucaena diets were productive at fattening bulls without adverse toxic effects.

8.4.1 Conjugation as a detoxification technique

This study provides evidence that conjugation with glucuronic acid is the major pathway in the detoxification of DHP in ruminants consuming high levels of leucaena. From the outset, it was apparent that most DHP was not being degraded by bacteria as mean urinary DHP concentrations of bulls monitored were persistently high. This occurred throughout the 4-week period, regardless of the length of time they had been consuming leucaena. Despite this, neither animal health nor productivity were negatively impacted; on the contrary, the LWGs of animals on high-leucaena diets were consistently high. Further, the results of the in-depth analysis of the excretory patterns of DHP revealed that almost all DHP is voided in the conjugated form. Therefore, complete conjugation of otherwise toxic levels of DHP had a profound detoxifying effect for the bulls.

The detoxifying effect of conjugation is related to the mode of toxicity of DHP which is due to the chelatory nature of the compound. The arrangement of the two *ortho* (*O*,*O*) moieties in DHP readily bind to divalent transition metals. This is biologically toxic in mammals as many of those metals, such as Zn, Mg, and Cu, are essential to regular cellular function (Berdoukas *et al.* 1993; Hoffbrand and Wonke 1997; Porter 1997; Kwiatkowski 2008). The effect of conjugation is to enzymatically attach an acidic compound to a hydroxyl group to inhibit chelation, effectively eliminating the biological activity of the toxin (Singh *et al.* 1992; Galanello 2007; Crisponi and Remelli 2008). Conjugation also increases the water solubility of the compound, increasing the speed of urinary clearance of toxin (Pang *et al.* 1994; Galanello 2007; Sooriyaarachchi and Gailer 2010; Dhakal *et al.* 2013).

Conjugation of DHP in ruminants has long been known to have occurred, as hydrolysis of the conjugate was a necessary step in the method for measuring free DHP levels (Hegarty *et al.* 1964a), although it was not historically considered a protective mechanism. The initial focus of DHP-toxicity was on its inhibition on thyroid hormones, even though it was reported that the conjugated form of DHP had little negative effect on the enzymes

involved in thyroid hormone synthesis (Christie *et al.* 1979). Interestingly, there was no regard for the possible role of conjugation in reducing toxicity. Much like the early work on mineral supplementation, after the discovery of *S. jonesii*, research into the protective properties of conjugation was largely ignored.

The original notion that bacterial degradation was the principal pathway of detoxification (Jones and Megarrity 1983), was also probably based on misinterpretation of why asymptomatic leucaena-fed goats in Hawaii were excreting "virtually no DHP" (Jones 1994). A crucial detail at the time was largely overlooked: the goats were in fact excreting 2,3-DHP and this was reported as a blue colour reaction to iron(III) chloride solution (Jones and Megarrity 1983). The original publication from this study (Jones 1981) however made no mention of this reaction, but it was later noted by Jones *et al.* (2009) as one of the first instances of 2,3-DHP detection. There have since been several misrepresentations of the occurrence of 2,3-DHP (Jones and Megarrity 1983; Ford *et al.* 1984; Jones *et al.* 1985a; Jones *et al.* 2009), all of which overlooked the significance of conjugation of DHP at the time of analysis.

8.4.2 High levels of 2,3-DHP

This study confirms the now persistent finding that 2,3-DHP is the end-point metabolite of mimosine degradation in ruminants. The breakdown of 3,4-DHP in the presence of *S. jonesii* has been widely studied and it has been reported in numerous studies to be converted to the isomer 2,3-DHP as an intermediate before complete degradation, despite infrequent detection *in vitro* (Jones *et al.* 1985a; Allison *et al.* 1987; Allison *et al.* 1990; Allison *et al.* 1992; Paul *et al.* 1999). This led researchers to assume that it was uncommon and transient, which is in conflict with the majority of recent findings where incomplete degradation of 3,4-DHP *in vivo*, resulted in high concentrations of 2,3-DHP (Ghosh *et al.* 2007; Dalzell *et al.* 2012; Phaikaew *et al.* 2012; Contreras-Hernández *et al.* 2013; Graham *et al.* 2013; Ruz-Ruiz *et al.* 2013; Halliday *et al.* 2014b; Halliday *et al.* 2014c) (Chapter 5). RNA PCR screening indicated that *S. jonesii* was present in many of these studies, although frequently at low populations. As such, it is unlikely that the accumulation of 2,3-DHP is the sole result of *S. jonesii* activity (McSweeney and Shelton 2009). Despite the continuous high levels of 2,3-DHP excreted in the leucaena-fed bulls in this study, this did not impact animal LWG production. This can largely be attributed to both the conjugation of DHP and the reduced toxicity of the 2,3-DHP isomer. The persistent identification of 2,3-DHP also confirms the herd spot samplings of previous work were correct interpretations of DHP status of the animals.

8.4.3 The conjugate of 2,3-DHP identified

The vast majority of 2,3-DHP excreted in bulls (which accounted for the vast majority of DHP excreted) was in conjugated form. The toxicity of DHP is due to the long-term disruption of homeostasis of essential metals (including Cu^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}) impacting metalloenzyme function (Puchała *et al.* 1995; B. Di Marco *et al.* 1999; Liu and Hider 2002; Flora and Pachauri 2010). This concept has been widely researched in human pharmacological research where 3,4-DHP is exploited for iron overload therapy (Santos 2008; Santos *et al.* 2012; Lachowicz *et al.* 2015). However, the compound is rapidly eliminated from the body after between 47–132 minutes due to conjugation (Galanello 2007; Flora and Pachauri 2010; Sooriyaarachchi and Gailer 2010). The conjugate form of DHP in the present study was identified as a glucuronide (bound with glucuronic acid). This is a common non-specific detoxification mechanism for dealing with a variety of xenobiotics, especially hydroxyl compounds (Gupta and Akbar 1993; Sahlu *et al.* 1995; Galanello 2007; Crisponi and Remelli 2008). It is this hepatic biotransformation of the ligand by glucuronidation that renders the DHP inert, with previous studies in humans also identifying up to 90% excreted as the 3-*O*-glucuronide conjugate (Singh *et al.* 1992; Flora and Pachauri 2010).

The conjugate form of DHP was identified in the HPLC chromatogram, with a shift from conjugated to free form with increasing acid hydrolysis. Of note is the finding that the methodology for all previous samplings has been incomplete in acid-hydrolysing urinary DHP, with ~70% remaining conjugated. Re-analysis of previous chromatograms from buffalo, goats and cattle from Australia and Indonesia confirmed the presence of large amounts of the 2,3-DHP-*O*-glucuronide via UV spectral analysis (data presented in previous chapters). While this finding infers that all previous work underestimated the amount of DHP excreted by over half, it also implies that any conjugated DHP was likely to be non-toxic. Unfortunately, due to the acid-preservation of these samples, it was not possible to determine the percentage of DHP conjugated at the time of collection.

Due to the strong detection of glucuronic acid conjugates, the primary conjugate of DHP is confirmed as the glucuronide form. The secondary conjugate is presumed to be that of a sulphate conjugate, which is also common (Pang *et al.* 1994; Shelnutt *et al.* 2002; Dhakal *et al.* 2013). The greatly reduced HPLC RT of the secondary conjugate is indicative of a much more polar compound, such as the sulphate form. The secondary DHP conjugate was present sporadically, and was not present in samples subjected to HDMS analysis. Thus, the presence of a sulphate form of conjugate is inconclusive and work is ongoing.

8.4.4 Findings regarding S. jonesii

The results of this study once again confirm that *S. jonesii* is simultaneously ubiquitous to ruminants with the high positive RNA PCR results, yet present in the rumen in low numbers with infrequent detection with DNA PCR indicative of low populations, at the limit of detection (Padmanabha *et al.* 2014). The assertion that *S. jonesii* is part of the normal flora of ruminants stems from the fact that it shows specificity for arginine and histidine as metabolisable substrates (Allison *et al.* 1992), with growth *in vitro* enhanced with the addition of these amino acids, as well as phenylalanine (McSweeney and Shelton 2009). This occurs at the expense of 3,4-DHP or 2,3-DHP utilisation. The unusual use of arginine and histidine as energy substrates is unique to *S. jonesii* [a strict anaerobic *Chemoorganotroph* (Allison *et al.* 1987; Allison *et al.* 1992; McSweeney *et al.* 2002)] and most likely explains why it is always detected in ruminants with the absence of leucaena in diet (Padmanabha *et al.* 2014). This affords it a unique, albeit limited, physiological niche in the rumen (McSweeney *et al.* 1993a), although the lack of specificity for DHP as preferred substrate may explain why, despite large amounts of DHP in the rumen, populations remain low in leucaena-fed bulls. In fact, other authors have indicated that *in vitro* degradation of the isomers of DHP by *S. jonesii* can be lost in the absence of a substrate (Domínguez-Bello *et al.* 1997), but also inhibited by an excess of the alternate isomer (Rincón *et al.* 1998; McSweeney and Shelton 2009). In addition, there is known isomer-specificity within the isolated strains of *S. jonesii* (Allison *et al.* 1992; Jones 1994; McSweeney and Shelton 2009), not accounting for the SNPs that have been identified in this thesis.

Without explicitly testing for it in this study, the accumulation of 2,3-DHP is likely due to several factors, including: critically low populations of *S. jonesii* (Hammond *et al.* 1989; Domínguez-Bello *et al.* 1997; McSweeney *et al.* 2002); strains of *S. jonesii* not able to degrade 2,3-DHP (Jones 1994); and competitive inhibition of DHP degradation due to the presence of more favourable amino acid substrates (McSweeney and Shelton 2009).

The findings that high levels of 2,3-DHP were present despite low populations of *S. jonesii* may suggest that a consortium of many morphological types of bacterial species and/or strains are involved in the conversion of 3,4-DHP to 2,3-DHP (Domínguez-Bello and Stewart 1990; Domínguez-Bello and Stewart 1991; Tan *et al.* 1994; Domínguez-Bello *et al.* 1997; Chhabra *et al.* 1998; McSweeney *et al.* 2002; McSweeney and Shelton 2009; Aung *et al.* 2011; Derakhshani *et al.* 2016). While little is known of the metabolic fate of 2,3-DHP, nor why isomerisation of 3,4-DHP occurs (Allison *et al.* 1994), it is suggested

that the degradation of 2,3-DHP likely follows similar pathways to the catabolism of related DHP compounds by aerobic microbes (Allison *et al.* 1994). There are many aerobic microorganisms known to degrade pyridine-compounds (Watson *et al.* 1974a), and thus there should be no surprise that biological pathways have evolved to degrade naturally occurring DHP compounds (Watson *et al.* 1974b), as they also commonly exist as intermediates in pyridine degradation.

The finding of the mono-hydroxypyridines 3,-HP and 4-HP in urine has not been recorded previously, and may provide insight as to the pathways by which 3,4-DHP is isomerised to 2,3-DHP. Whilst the toxicities of these monohydroxypyridines is not certain, they also were found to be highly conjugated when excreted.

8.5 Conclusion

This study has highlighted the high value of leucaena as a forage for fattening, and confirmed the new paradigm of toxicity amelioration in ruminants via a process of hepatic conjugation. The attachment of a glucuronic acid conjugate to DHP effectively neutralises the biological toxicity of the otherwise strong ligand, preventing essential element deficiencies. This occurred with all bulls and with almost all DHP. Bulls newly introduced to high levels of leucaena also demonstrated this same proficiency for conjugation as those on long-term 100% leucaena diets.

Despite the apparent ubiquitous presence of *S. jonesii* in bulls tested, the implied low populations cannot be sufficient to degrade all DHP *in vivo* and indeed this was the case in this study, confirming consistently similar findings through this thesis. The longitudinal profile of DHP excretion obtained in this study also confirms that the high levels of 2,3-DHP in mean herd spot urine tests are not transient and are valid assessments of the usual metabolism of mimosine, whereby 2,3-DHP is the majority endpoint, albeit in a conjugated form.

It is recommended that there should be a redefinition of how DHP-toxicity is assessed, with the level of conjugation a vital parameter in the determination. Further work should include improved spot-urine testing using iron(III) chloride to estimate the level of conjugation of DHP. The role of mono-HP compounds as possible intermediates in the isomerisation of 3,4-DHP to 2,3-DHP should also be investigated, as well as the indication of a possible sulphate conjugate of DHP.

Chapter 9 Conclusions and future directions

9.1 Conclusions

Despite the many nutritional values of leucaena, the issue of toxicity, first identified in Australia in the 1970s, has been an ongoing barrier to wide-scale adoption for ruminant feeding in the tropical world. The discovery of the hydroxypyridone (DHP) degrading bacterium *Synergistes jonesii* in the mid 1980s, and subsequent commercialisation of a bacterial inoculum, was thought to overcome this issue. However, since 2003, concerns have been raised over the efficacy and persistence of the inoculum in Australia, and of the occurrence of the bacterium in other tropical countries. The research presented in this thesis has led to significant advances in microbiological identification and quantification of *S. jonesii*, and in DHP chromatographic measurement. The main objective of this thesis was to study protective mechanisms against toxicity, including the efficacy of microbial degradation of DHP by *S. jonesii*, and to identify other mechanisms such as metabolic detoxification. The results of this thesis have greatly advanced our understanding of leucaena toxicity, many of which are in dissensus with original views. This has led to a new paradigm for toxicity management and the following conclusions.

9.1.1 Findings regarding DHP

Historically, 3-hydroxy-4(1*H*)-pyridone (3,4-DHP) was considered the primary metabolite of mimosine post-ingestion in ruminants and was described as a potent goitrogen, inhibiting the iodination of tyrosine-based hormones due to its analogous nature with pyridoxal which antagonises peroxidase-based enzyme pathways. The secondary isomer 3-hydroxy-2(1*H*)-pyridone (2,3-DHP) — often incorrectly referred to as 2,3-dihydroxypyridine — was originally detected infrequently in urine and rarely in *in vitro* degradation studies, and as such, it was thought to exist as merely a transitory intermediate isomer.

However, a less studied aspect of DHP was the chelatory nature of these compounds as ligands, binding with essential metals and leading to deficiencies. Indeed, the clinical signs of DHP-toxicity in ruminants can more accurately be ascribed to severe essential element deficiencies. Whilst research in the field of DHP toxicity in ruminants largely considered the goitrogenic effects, a largely unacknowledged body of work, published during the same time period investigating pharmacological uses of DHP, strongly supports the toxic chelatory nature of DHP, leading to the conclusion that DHP acts primarily as a potent ligand, chelating with divalent metal cations *in vivo*. This is due to the arrangement of DHP as the keto-enol tautomer at physiological pH, with the hydroxy-carbonyl functional moiety

on the pyridine ring forming strong bidentate coordinate-covalent bonds with a central metal ion, which is subsequently voided in the urine.

A consistent finding throughout all studies in this thesis was that high levels of DHP are excreted when ruminants consume high leucaena diets and that almost all DHP excreted is of the isomer 2,3-DHP. As such, 2,3-DHP can now be considered the common end-point metabolite of mimosine degradation in ruminants on high leucaena diets. Despite high levels of 2,3-DHP excreted, there was no evidence of deleterious impacts on animal health or production observed throughout all studies. A contributing factor to this is that, contrary to initial views that both isomers were equally toxic, when considering the chelatory capacity, 2,3-DHP is in fact an order of magnitude less toxic than 3,4-DHP. This is due to the re-arrangement of the electronegative carbonyl moiety significantly closer to the electropositive ring nitrogen, greatly reducing the affinity to bind to cationic metals.

This conversion of 3,4-DHP to 2,3-DHP may in fact be a synergistic coping response with the evolutionary advantage of isomerisation considered two-fold: for the ruminant, it reduces the toxic chelatory capacity; whereas for the microbial populations, it results in a weaker structure to break down.

The conversion pathway of 3,4-DHP to 2,3-DHP is as yet unknown. *S. jonesii* is known to be capable of this, and was commonly detected in ruminants in all studies of this thesis, however it was often at or below the levels of detection, indicating low populations which are unlikely to be solely capable of this isomerisation when 100% diets of leucaena are consumed.

Whilst not conclusive, this thesis has contributed to the advancement of this pathway, with the previously undescribed hydroxypyridines 3-HP and 4-HP commonly detected. This may suggest microbial isomerisation, at least in part, occurs via stepwise reductive reactions prior to re-hydroxylation. This is a common pathway in many pyridine-degrading bacteria which destabilises the compound, more easily enabling hydrolytic ring fission.

9.1.2 Findings regarding S. jonesii

The results of this thesis support the new notion that *S. jonesii* is ubiquitous in all ruminants and is not dependent on previous exposure to leucaena. Whilst this indicated that inoculation was not required to introduce *S. jonesii* into ruminants newly consuming leucaena, there were several provisos: (a) there were many molecular variants of *S. jonesii* (often associated with differing geographical locations), due to the presence of

point mutations in the genetic sequence, likely affecting functional DHP-degrading capacity; and (b) *S. jonesii* was always present at low population density.

In the absence of inoculation as a management strategy, there was evidence that indigenous populations of *S. jonesii* were likely contributing to partial, yet incomplete, degradation of DHP and likely also contributing to the conversion of 3,4-DHP to the less toxic isomer 2,3-DHP. It is not known to what extent other microbial populations may also be contributing to this conversion. Also, whilst high levels of DHP are commonly measured in urine, it is not clear what proportion of ingested DHP (mimosine) this represented. As such, although the capacity to fully degrade DHP appears limited, the role of *S. jonesii* in total animal protection cannot be excluded, and further work, such as DHP recovery studies with the new methodologies should aim to apportion a relative significance on the ruminal degradation and isomerisation of DHP in addition to metabolic conjugation. It is however concluded that, whether animals received inoculum or not, *S. jonesii* alone is not capable of affording complete protection to ruminants on high leucaena diets.

9.1.3 Findings regarding metabolic detoxification via conjugation

The findings of this thesis strongly support the pathway of conjugation as a crucial method in the neutralisation of DHP toxicity for ruminants consuming leucaena. The phenomenon of asymptomatic and highly productive ruminants consuming sole leucaena diets, despite excreting high levels of undegraded 2,3-DHP, was commonly observed in all experiments. This can be attributed primarily to the fact that almost all DHP (and associated HP) was excreted in conjugated form, with the primary conjugate identified as glucuronic acid. The resulting DHP-3-*O*-glucuronide complex has a severely reduced ligand strength, and an increased water-solubility enabling it to be more rapidly excreted from the body (Galanello 2007; Flora and Pachauri 2010; Sooriyaarachchi and Gailer 2010). Ruminants were shown to quickly adapt to high leucaena diets as the process of glucuronidation is a non-specific metabolic mechanism for neutralising a variety of toxins.

9.1.4 A new paradigm

The results of this thesis have contributed new insights into the detoxification mechanisms ruminants employ to deal with the leucaena toxin DHP, of which a critical pathway is that of hepatic conjugation. Conjugation was shown to be highly efficient and effective in all ruminant species. This approach is not novel, however its importance is only now being fully appreciated.

This thesis showed that 100%-leucaena diets were successful in providing a low-cost, low-labour feed source for the productive fattening of bulls for smallholder farmers in Indonesia. These findings have great importance to livestock production in the tropical world, as ruminants are inherently and autonomously capable of detoxifying mimosine (after a brief adaptation period) and its metabolites, without negatively affecting their heath, performance, or production. This represents the removal of a world-wide barrier to adoption of leucaena-feeding for farmers.

This has led to a new paradigm in animal management and new research opportunities, however is in dissensus with many reports from earlier leucaena toxicity research. Where possible, the discordant results of this thesis are ascribed to likely reasons and remaining knowledge gaps acknowledged. These include:

The lack of incidence of enlarged thyroids and/or decreased thyroid hormones

The goitrognic effect of DHP and associated symptoms were widely reported in early research, however, were not observed in the studies of this thesis; other authors published similar findings (Hamilton *et al.* 1971; Falvey 1976). It is hypothesised that the conversion of 3,4-DHP to 2,3-DHP and subsequent conjugation prevented toxicity from manifesting. Whilst goitre was commonly reported on in early research, anecdotal instances of goitre from graziers and smallholder farmers are rare and when present, compounding factors such as pre-existing essential metal deficiencies and/or an increased susceptibility of individual animals may contribute to the severity of presentation. The age of the animal, breed, and seasonal conditions (Jones *et al.* 1976) can also have an effect on susceptibility. The common practice of supplementing animals with mineral blocks/licks would also alleviate any possible metal deficiencies DHP toxicity may cause, and contribute to the prevention of clinical symptoms.

The lack of significant levels of 3,4-DHP in urine

Whist studies conducted during the 1980s reported 3,4-DHP as the common isomer excreted in ruminants consuming leucaena, in most cases there were low to negligible levels of 3,4-DHP measured in the studies of this thesis, with the vast majority excreted as 2,3-DHP. Possible causes for this isomerisation to 2,3-DHP include the numerous bacteria that have been shown to specialise in degradation of either isomer (Hammond *et al.* 1989; Domínguez-Bello and Stewart 1990; Domínguez-Bello and Stewart 1991; Tan *et al.* 1994; Domínguez-Bello *et al.* 1997; Chhabra *et al.* 1998; Aung *et al.* 2011; Derakhshani *et al.* 2016), including different strains of *S. jonesii* (Allison *et al.* 1992). Indeed there were many variants of *S. jonesii* detected in the studies of this thesis, which are likely to affect the

capacity to degrade DHP isomers. While the different, and possibly changing, strains of *S. jonesii* may be responsible for the change in the dominant isomer excreted, there has historically been a general misunderstanding and misrepresentation of the isomer 2,3-DHP (Jones 1981; Ford *et al.* 1984; Jones *et al.* 1985a; Jones 1994; Jones *et al.* 2009). Indeed, the seminal study of Jones and Megarrity (1983) initially overlooked the finding of 2,3-DHP in urine. The lack of detection of 2,3-DHP in *in vitro* degradation studies would have also contributed to the reduced emphasis on the isomer. In addition, in early studies, detection of DHP was conducted by colorimetric assessment with iron(III) chloride solution, which can be confounded by incomplete hydrolysis of conjugated DHP, and the presence of other polyphenolic compounds if not removed (Megarrity 1978; Lowry *et al.* 1985). Considering HPLC analysis, the more polar 3,4-DHP elutes early in the chromatogram and can more easily be confounded by the presence of other polar urinary metabolites absorbing at that wavelength. While 2,3-DHP elutes much later and is far more easily identifiable, if not completely hydrolysed, it will have a reduced retention time compared to a 2,3-DHP standard which could easily have been overlooked.

The reduced efficacy of inoculation with *S. jonesii*

The results of this thesis did not show a rapid and complete degradation of DHP after inoculation, in contrast with many earlier studies. In addition, when *S. jonesii* was present, animals on high leucaena diets continued to excrete undegraded DHP. As such, it is possible some degradation was occurring by indigenous *S. jonesii* strains which prevented an observable effect of the inoculated *S. jonesii*. Dosing with the fermenter cultured inoculum was shown to influence the rate of degradation of different isomers however.

The low populations of *S. jonesii* in the rumen suggest it alone is not capable nor responsible for complete DHP degradation, especially when animals are consuming high levels of leucaena. An explanation for the more dramatic differences in DHP excretion after ruminal inoculation in Australia may be attributed to the lower levels of leucaena in diet, typical of Australian grazing systems, combined with the inoculation of variants of *S. jonesii* with greater functional degradation capacity.

There are many anecdotal reports from graziers, of steers improving in condition and recovering from toxicity symptoms such as hair loss and excessive salivation after inoculation in Australian grazing systems. These signs of initial mimosine toxicity commonly abate within the first 4 weeks, regardless of inoculation and are also commonly reported by Indonesian farmers. The coincidence of the recovery from mimosine toxicity

and the inoculation of animals in Australian grazing systems could be mistaken for the effects of inoculation.

The prevalence of conjugation

A major finding of this thesis was the role of conjugation in detoxification of DHP, however this finding was not novel and was given consideration as a detoxification mechanism in early research into leucaena toxicity (Christie *et al.* 1979; Hegarty *et al.* 1979). Indeed the original method for determining DHP in urine acknowledged the conjugate and the need to hydrolyse samples for accurate colorimetric assessment (Hegarty *et al.* 1964a).

After microbial degradation of DHP was postulated in 1981 (Jones 1981) and the subsequent discovery of *S. jonesii* in 1984 (Jones and Lowry 1984), research into DHP toxicity in ruminants converged with a strong focus on microbial degradation, with little consideration for the role of conjugation. As largely all measurement of urinary DHP from 1984 onwards was conducted on acid-hydrolysed samples, utilising a method that has been shown to be incomplete, the extend of conjugation would not have been detected.

9.1.5 Practical animal recommendations

The practical outcome of this research is the identification that high levels of leucaena are safe to consume in Indonesian smallholder bull fattening systems and that conjugation of undegraded DHP plays an important role in this. In order to manage the initial mimosine toxicity commonly experienced, a graduated introduction to leucaena is recommended for these systems.

The results also suggest that inoculation in Australian grazing systems may not be as effective as originally postulated, and indeed may no longer even be required, due to the presence of indigenous DHP-degrading bacteria and the effect of conjugation neutralising the toxicity of undegraded DHP. Whilst not explicitly tested, all indications are that conjugation occurs in Australian cattle at similar rates as in Indonesian ruminants.

The conclusions of this thesis herald a revision of current recommendations to Australian graziers whereby inoculation may no longer be necessary. Further investigation into the remaining knowledge gaps, identified as future research needs, is important before such a dramatic change in practice is advised.

It is however, strongly recommended that herd urine testing be conducted in order to elucidate the protection strategies being employed, whereby strategic inoculation may still

play a role in certain circumstances. Maintaining available mineral supplements and ready access to water, especially in Indonesian systems, is also recommended.

9.2 Future research needs

Despite the findings of this thesis, there remain topics of further research in order to fully appreciate this new paradigm, and to inform decisions to change current practices. Initially, testing of Australian animals consuming leucaena should be conducted to confirm the efficacy of conjugation domestically. This would involve improving chromatographic methodologies: combining liquid chromatography with UV absorption, with a volatile mobile phase suitable for mass spectroposcy. The use of LCMS would allow the secondary conjugate to be identified, hypothesised to be a sulphate conjugate. This would allow the most accurate assessment of DHP toxicity in ruminants.

Before management techniques can be updated, the overall significance of conjugation and degradation is required. Research to achieve this would involve metabolic recovery trials with a N isotope labelled mimosine infusion, allowing the fate of mimosine to be mapped throughout the circulatory system and excreta. This would also assist in understanding the isomerisation pathways of 3,4-DHP to 2,3-DHP.

As mimosine was not present in urine samples analysed, it was not the focus of this thesis. However, signs of alopecia and excessive salivation, ascribed to the antimitotic and chelatory effect of mimosine, were commonly observed in bulls upon their initial introduction to leucaena, especially when fed at 100% of diet. This is supported by strong anecdotal accounts from many farmers. As the effects are short-term in nature, mimosine is not considered an issue in animals fattened for human consumption, however, further research should consider the implications of mimosine on the reproductive performance of stud bulls or gestating cows when leucaena is first introduced.

Additional knowledge gaps that require further research include: the isomerisation pathway/s of 3,4-DHP to 2,3-DHP, which would involve the assessment of intermediates such as HP in the rumen and in circulation; and, the potential effect of DHP in circulation prior to conjugation, which would involve comparing the levels of free and conjugated DHP in the portal and hepatic veins.

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Chapter 10 References

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Appendix 1: Animal Ethics Approval Certificate SAFS/144/11/ACIAR

THE UNIVERSITY OF QUEENSLAND

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

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

ANIMAL ETHICS APPROVAL CERTIFICATE

Date: 28-Jul-2011

Dear Dr Max Shelton, Agriculture and Food Sciences

The following project: *Investigations into DHP toxicity in livestock consuming leucaena in Queensland and eastern Indonesia and the potential presence of new DHP degrading bacterium or alternate strains of Synergistes*

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

AEC Approval Number: SAFS/144/11/ACIAR

Previous AEC Number:

Approval Duration: 01-Aug-2011 to 01-Aug-2014

Permit(s):

SUBSPECIES	STRAIN	CLASS	GENDER	SOURCE	AMOUNT
Sheep	Mixed	Other	Mix	Privately owned	50
Goats		Other	Mix	Privately owned	100
Other Domestic Mammals	Buffalo	Other	Mix	Privately owned	50
Cattle		Other	Mix	Privately owned	300

Proviso(s):

The committee directed the CI to nominate a person who is not directly involved in the project , at the Indonesia site, to provide reports and monitor the project on behalf of the committee as per The Code 2.2.43 and 2.2.29.
i) The CI is required to ensure that all documentation required overseas is obtained.
ii) That the CI withdraws from the project if the welfare of the animals falls below an equivalent acceptable standard to that which the committee would normally approve.

Please note the animal numbers supplied on this certificate are the total allocated for the approval duration

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

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

This certificate supercedes all preceeding certificates for this project (i.e. those certificates dated before 28-Jul-2011

Page 1 of 1

Appendix 2: Animal Ethics Approval Certificate LCAFS/035/10

THE UNIVERSITY OF QUEENSLAND

PLEASE KEEP THIS FORM AS YOUR RECORD OF YOUR AEC APPROVAL NUMBER

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

ANIMAL ETHICS APPROVAL CERTIFICATE Date: 10-May-2010

Dear Dr Max Shelton, Land, Crop and Food Sciences

The following project: *Investigations into degradation of mimosine by Synergistes jonesii in beef cattle herds grazing leucaena in Queensland*

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

AEC Approval Number: LCAFS/035/10

Previous AEC Number:

Approval Duration: 10-May-2010 to 10-May-2011

Permit(s):

SUBSPECIES	STRAIN	CLASS	GENDER	SOURCE	AMOUNT
Cattle	Beef	Juvenile/Weaners/Pouch animal	Male	Privately owned	20

Proviso(s):

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

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

This certificate supercedes all preceeding certificates for this project (i.e. those certificates dated before 10-May-2010

Page 1 of 1

Appendix 3: Animal Ethics Approval Certificate AFS/160/12/ACIAR

THE UNIVERSITY OF QUEENSLAND

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

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

ANIMAL ETHICS APPROVAL CERTIFICATE

Date: 02-May-2012

Dear Dr Max Shelton, Agriculture and Food Sciences

The following project: *Investigations into the effect of 2,3- DHP toxicity, common in goats fed leucaena in Indonesia*

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

AEC Approval Number: AFS/160/12/ACIAR

Previous AEC Number:

Approval Duration: 03-May-2012 to 03-May-2013

Permit(s):

SUBSPECIES	STRAIN	CLASS	GENDER	SOURCE	AMOUNT
Goats		Other	Mix	Privately owned	40

Proviso(s):

i) The CI is required to ensure that all documentation required overseas is obtained.
ii) That the CI withdraws from the project if the welfare of the animals falls below an equivalent acceptable standard to that which the Committee would normally approve.

Please note the animal numbers supplied on this certificate are the total allocated for the approval duration

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

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

This certificate supercedes all preceeding certificates for this project (i.e. those certificates dated before 02-May-2012

Page 1 of 1

Appendix 4: Animal Ethics Approval Certificate SAFS/023/15/ACIAR

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

UQ Research and Innovation
Director, Research Management Office
Nicole Thompson

Animal Ethics Approval Certificate

28-Apr-2015

Please check all details below and inform the Animal Welfare Unit within 10 working days if anything is incorrect.

Activity Details

Chief Investigator:	Dr Max Shelton
Title:	Investigations into DHP toxicity in livestock consuming leucaena in Queensland and eastern Indonesia
AEC Approval Number:	SAFS/023/15/ACIAR
Previous AEC Number:	SAFS/420/14/ACIAR
Approval Duration:	28-Apr-2015 to 28-Apr-2016
Funding Body:	ACIAR
Group:	Production and Companion Animal
Other Staff/Students:	Michael Halliday, Tanda Panjaitan, Chris McSweeney, Dr Dahlanuddin, Debbie Kana Hau, Jacob Nulik
Location(s):	Other International Location

Summary

Subspecies	Strain	Class	Gender	Source	Approved	Remaining
Cattle		Adults	Mix	Privately owned	380	380
Cattle	Water Buffalo (Bubalus bubalis)	Adults	Mix	Privately owned	45	45
Goats		Adults	Mix	Privately owned	100	100

Permits

Provisos

The committee directed the CI to nominate a person who is not directly involved in the project , at the Indonesia site, to provide reports and monitor the project on behalf of the committee as per The Code 2.2.43 and 2.2.29.
i) The CI is required to ensure that all documentation required overseas is obtained.
ii) That the CI withdraws from the project if the welfare of the animals falls below an equivalent acceptable standard to that which the committee would normally approve.

Approval Details

Description	Amount	Balance
Cattle (Mix, Adults, Privately owned)		
28 Apr 2015 Initial approval	380	380
Cattle (Water Buffalo (Bubalus bubalis), Mix, Adults, Privately owned)		
28 Apr 2015 Initial approval	45	45
Goats (Mix, Adults, Privately owned)		
28 Apr 2015 Initial approval	100	100

Animal Welfare Unit
UQ Research and Innovation
The University of Queensland

Cumbrae-Stewart Building
Research Road
Brisbane Qld 4072 Australia

+61 7 336 52925 (Enquiries)
+61 7 334 68710 (Enquiries)
+61 7 336 52713 (Coordinator)

animalwelfare@research.uq.edu.au
uq.edu.au/research

Appendix 5: Animal Ethics Approval Certificate SAFS/038/12/ACIAR

THE UNIVERSITY OF QUEENSLAND

UQ Research and Innovation
Director, Research Management Office
Nicola Thompson

ANIMAL ETHICS APPROVAL CERTIFICATE

25-Mar-2013

Activity Details

Chief Investigator: Dr Max Shelton, Agriculture and Food Sciences

Title: Study of growth path of Bali cattle fed leucaena/sesbania from weaning to market

AEC Approval Number: SAFS/038/12/ACIAR

Previous AEC Number:

Approval Duration: 26-Mar-2012 to 26-Mar-2015

Funding Body:

Group: Production and Companion Animal

Other Staff/Students: Chris McSweeney, Tanda Panjaitan, Jacob Nulik, Debora Kana Hau, Dr Dahlanuddin, Michael Halliday, Resti Gabriela Edison, Charles Pakereng, Dessy N Liubana

Location(s): Other International Location

Summary

Subspecies	Strain	Class	Gender	Source	Approved	Remaining
Cattle	Bali	Juvenile / Weaners / Pouch animal	Mix	Privately owned	1080	1080

Permit(s):

Proviso(s):

Proviso for overseas work:

i) The CI is required to ensure that all documentation required overseas is obtained.
ii) That the CI withdraws from the project if the welfare of the animals falls below an equivalent acceptable standard to that which the committee would normally approve.

Approval Details

Description	Amount	Balance
Cattle (Bali, Mix, Juvenile / Weaners / Pouch animal, Privately owned)		
26 Mar 2012 Initial approval	80	80
20 Mar 2013 Modification #1	1000	1080

UQ Research and Innovation
The University of Queensland
Cumbrae-Stewart Building
Research Road
Brisbane Qld 4072 Australia
T +61 7 3365 2925 (Enquiries)
T +61 7 3365 2713 (Manager)
F +61 7 3365 4455
E animalwelfare@research.uq.edu.au
W www.uq.edu.au/research/rid/