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The 22nd International Grassland Congress (Revitalising Grasslands to Sustain Our Communities) took place in Sydney, Australia from September 15 through September 19, 2013.

Proceedings Editors: David L. Michalk, Geoffrey D. Millar, Warwick B. Badgery, and Kim M.

Broadfoot

Publisher: New South Wales Department of Primary Industry, Kite St., Orange New South Wales, Australia

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

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Abstract. The tree legume *Leucaena leucocephala* (leucaena) is a high quality ruminant feed, vitally important for livestock production in the tropics despite the presence of mimosine in the leaves. This toxic non-protein amino acid has the potential to limit productivity and adversely affect the health of animals. The discovery and subsequent distribution in Australia of the ruminal bacterium *Synergistes jonesii* as an oral inoculum was shown in the 1980s to overcome these toxic effects. However, recent surveys of the status of toxicity worldwide; improved understanding of the chemistry and mode of action of the toxins; new techniques for molecular sequencing; and concerns about the efficacy of the *in vitro* inoculum; have cast doubt on some past understanding of leucaena toxicity and provides new insights into the geographical spread of *S. jonesii*. There is also confusion and ignorance regarding the occurrence and significance of toxicity in many countries worldwide. Ongoing research into the taxonomy and ecology of the *Synergistes* phylum, improved methods of inoculation, improved management solutions, along with awareness-raising extension activities, are vital for the future success of leucaena feeding systems.

Keywords: *Leucaena leucocephala*, *Synergistes jonesii*, toxicity, mimosine, dihydroxypyridine, DHP, urine.

Introduction

Leucaena is a multipurpose forage tree legume widely used in the tropical world (Shelton and Brewbaker 1994) with 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). Whilst 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 provides edible forage long into the dry season (Shelton and Brewbaker 1994). Leucaena is a vitally important source of protein for ruminants throughout Southeast 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; there are currently over 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 product, high cost of protein concentrates, and shortage of nitrogen for tropical grass pastures will increase the need for alternative high protein feed sources in the tropics. This is leading to increased leucaena plantings 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 leaf and young pods (up to 9% dry matter (DM) in young leaves and 4-7% DM in seeds) (Hegarty *et al.* 1964b). 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.* 1964b; Jones *et al.* 1976; Allison *et al.* 1992; Jones 1994). They found that mimosine was rapidly converted to 3,4-dihydroxypyridine (3,4-DHP) post-ingestion resulting in reduced feed intake, decreased live weight 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 1980's led to the isolation and identification of a previously unknown, and at the time unusual species of bacterium from the rumen of an Hawaiian goat which 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 provided protection against the toxic effects of

leucaena by degrading DHP to harmless by-products (Jones and Megaritty 1986). This success was followed by the development of a commercial inoculum in Australia, comprising initially of rumen fluid (*in vivo*) and subsequently of fermenter-produced (*in vitro*) mixed inoculum containing *S. jonesii* (Klieve *et al.* 2002). Since animals were reported to readily transfer the 'bug' within a herd (Quirk *et al.* 1988; Pratchett *et al.* 1991) (the exact methods of which are unknown, but likely encapsulated in saliva or faeces) it was recommended that only 10% of the herd need be inoculated. Protection of herds from toxicity in this way has led to annual live weight gains 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 thought to be resolved in Australia. However, a survey of the protection status of herds in Queensland on high leucaena diets has shown 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. There was ignorance of both methods of detection of toxicity and of management to limit adverse effects. This was of great concern as undiagnosed subclinical toxicity leads to reduced animal production (Dalzell *et al.* 2012).

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

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 over 13 other countries were not protected. He hypothesised 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 in those countries protected from toxicity there was greater use of leucaena as forage for ruminant production (Jones 1981).

However, detailed recent monitoring of ruminant leucaena toxicity has demonstrated a more complex situation. Using the criteria that mean urinary DHP levels in protected herds should be < 100 mg/L (Dalzell *et al.* 2012), a survey of 20 villages within 4 islands of eastern Indonesia found that village herds within close proximity (<40 km and in some cases <1 km) differed in their protection status (Halliday *et al.* 2013b). It is therefore unwise to classify the protection status of whole countries. Furthermore, as leucaena feeding in the tropics increases, with movement of animals with DHP degrading bacteria both in and out of villages where leucaena is fed, this may see some ruminants lose protection, while others in neighbouring villages may gain protection if their animals are maintained on leucaena.

Thailand's inclusion as a protected country (Jones 1994) was based on the lack of urinary DHP found 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 farmer level.

Confusion regarding toxicity symptoms

When leucaena was first introduced to Australia, the clinical symptoms of toxicity were quite serious, presenting as emaciated animals, with some animal mortalities (Jones *et al.* 1978). However, severe clinical symptoms are now rarely seen, and most farmers feeding leucaena are ignorant of the 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 suffering from toxicity, as data are not available. Also, since their animals fed leucaena may still be performing better than those without legume, there is understandable confusion over the toxicity 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.* 2013b) including no indication of goitre (Palmer *et al.* 2010). The reasons for this are not understood but may be due to chemical conjugation of the toxins reducing their toxicity (mentioned later in this paper).

The symptoms associated with leucaena toxicity are now discussed and clarified. They are ascribed to both mimosine and its primary metabolites: 3,4-DHP and 2,3-DHP (Fig. 1). While structurally similar, these toxins have different modes of toxicity. Although there is some overlap, they are responsible for different clinical, and subclinical symptoms. It is therefore essential that toxicity symptoms are understood and the causes correctly

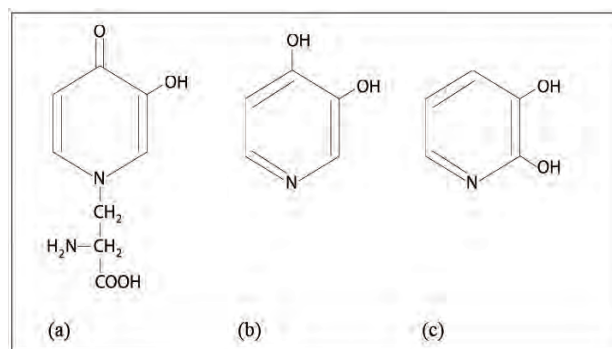


Figure 1. (a) Mimosine and its ruminal degradation products; (b) 3,4-DHP and (c) 2,3-DHP, adapted from Hammond (1995).

identified for proper management of leucaena-based feeding systems.

Mimosine toxicity

Mimosine (β -[*N*-(3-hydroxy-4-oxopyridyl)]- α -amino-propionic acid) (Hegarty *et al.* 1976) is acutely antimitotic, inhibiting 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 (Ram *et al.* 1994), oesophageal lesions (Jones *et al.* 1978), foetal abortions (Holmes 1980), low bull fertility (Holmes 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 is a tyrosine analogue (Hegarty *et al.* 1964a; Hashiguchi and Takahashi 1977), capable of inhibiting enzyme functions such as tyrosine decarboxylase and tyrosinase (Crouse *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 on 100% leucaena (Hegarty *et al.* 1964a). Hair loss is commonly observed from the penis sheath and tail areas where growth is more continuous; mimosine only affects the follicle bulb in the active phase of growth (Hegarty *et al.* 1964a). This is best demonstrated in sheep as wool growth is largely continuous and therefore sensitive to alopecia from mimosine toxicity (Hegarty *et al.* 1964a). Alopecia is evident when levels of mimosine in diet are > 0.015% body weight (BW) (Szyzka and Termeulen 1985). As consumption of 100% fresh leucaena leaves may be as high as 0.031% BW mimosine (Tangendjaja *et al.* 1985) this would suggest 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 only appears when animals are first introduced to high leucaena diets (Ghosh *et al.* 2007; O'Reagain 2009). Ruminants typically acquire the ability to fully degrade mimosine in high leucaena diets (>50%) 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 2009). For this reason, mimosine toxicity symptoms rarely persist within an animal, especially if introduced to leucaena slowly (Jones 1979).

The degradation of mimosine to 3,4-dihydropyridine (3,4-DHP) occurs via many endogenous rumen bacteria (Hegarty *et al.* 1964a) and by plant hydrolase activity of endogenous enzymes within 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, Cu and Zn, inhibits many enzymatic pathways; these metals are also required for normal hair growth (Hegarty *et al.* 1964a; 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 (Puchala *et al.* 1995).

DHP toxicity

The primary metabolite of mimosine is the compound 3-hydroxy-4(1H)-pyridine (3,4-DHP) (Hegarty *et al.* 1976), which in the presence of certain ruminal microbes, can be further converted to its isomer 2,3-dihydropyridine (2,3-DHP) (D'mello 1992). DHP acts as a potent goitrogen, due to its 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 thyroxine (T₄) and triiodothyronine (T₃), resulting in depressed serum T₄ levels, which causes overstimulation and enlargement (up to 4 times) of the thyroid glands (goiter) (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, confirming the anti-thyroid effects of DHP (Hegarty *et al.* 1979). The effect of lowered serum T₄ and T₃ levels is reduced appetite, and ultimately a decreased LWG. Serum T₄ levels below 13 n mol/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 inhibits the trapping step rather than the binding step, as in the thyroid (Koutras *et al.* 1967; Harden *et al.* 1968), leading to excessive salivation after prolonged exposure to leucaena (Jones *et al.* 1976; Jones *et al.* 1978; Holmes 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 minerals (Ghosh and Samiran 2007). A deficiency in Zn was shown to be responsible for skin lesions (Mills 1978; Paul 2000), increased salivation (Mills 1978; Puchala *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 effects of DHP toxicity are 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 a 0-0.2 kg/head/day (Jones and Bray 1983). These lowered LWGs were found to be directly related to lowered levels of serum T₄, with a threshold of 25 n mol/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 of time that 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.* 2013b).

The dominance of 2,3-DHP

The isomer 2,3-DHP was originally thought to be transitory, and indicative of incipient ruminal colonisation of *S. jonesii* (Ford *et al.* 1984; Jones *et al.* 2009). Much recent data from Australia, Thailand and Indonesia contradicts this notion of 2,3-DHP being transitory (Dalzell *et al.* 2012; Phaikaew *et al.* 2012; Graham *et al.* 2013; Halliday *et al.* 2013b) and indicates 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 can be fatal given intraruminally in pure form (Puchala *et al.* 1995).

In a controlled cattle feeding trial in India (Ghosh *et al.* 2007), 2,3-DHP was the dominant isomer 21 days after commencement of leucaena feeding in cattle naïve to *S. jonesii*, as was the case for animals offered 25 to 100% leucaena in a controlled trial in Queensland (Halliday *et al.* 2013a). In the latter trial, all steers on diets of 25 to 100% leucaena were excreting 2,3-DHP at levels above 100 mg/L after only 2 weeks on leucaena, increasing through to week 7 when it formed the majority of the DHP excreted.

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: (1) rumen microbes other than *S. jonesii* are capable of degrading 3,4-DHP to 2,3-DHP; (2) the *in vitro* *S. jonesii* inoculum released in Australia may have lost/mutated the strains effective at complete DHP degradation; or (3) there are other environmental factors (including regulation of genes involved in DHP metabolism) affecting optimal ability of *S. jonesii* to completely degrade all DHP.

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 10³ cells/ml; however, a realistic PCR amplification of genomic DNA from rumen fluid/digesta is between 10⁴ and 10⁵ 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 by several logs. 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 was conducted of animals consuming diets of 0%-100% leucaena to understand the distribution and changes at the molecular level of *S. jonesii*. Rumen fluid or faeces was collected from cattle in Queensland and from cattle, sheep, goats, buffalos, 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.

The survey revealed that *S. jonesii* is far more widespread and indigenous to the rumen than originally realized and that many molecular variants of the type strain exist. The molecular detection of this bacterium internationally in ruminants, irrespective of their leucaena feeding status, corroborates some of the findings of Jones (1994), reporting on the inconsistent DHP degrading capability of rumen fluid from animals from cross-continental sites.

Yet, this information on its ubiquity has come with a proviso, that our sequence analysis from Australia and other countries showed SNPs, across ~800/1500 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' & '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 Vietnam cattle and goats). Among all the international samples analysed,

Jinnan cattle, Tibetan yak and Indonesian buffalos were 100% identical to the type strain of *S. jonesii*. Interestingly, the buffalos 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, thus, 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 is similarly fraught with ambiguity relating to the sensitivity and specificity of the quantitative real-time PCR (qPCR) technique. Compared to the 2-step nested PCR which produces ~800 bp long product, 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 the *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. These variants may potentially be responsible for the partial DHP degradation results.

Management of toxicity

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 developing country farmers. 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 with a glucose molecule. HPLC analysis with a reduced flow rate has been able to separate free DHP from conjugated DHP-glucoside. However preservation of urine prior to analysis requires strong acidification, which hydrolyses all conjugated DHP (Tangendjaja and Wills 1980) unless analysis is conducted immediately.

Whilst assay of serum T₄ and T₃ can be used as an indicator of DHP toxicity, (Jones *et al.* 1978; Megarritty and Jones 1983; 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 goiter 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.

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.* 1985). 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 Megarritty 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 Megarritty 1986). Rumen fluid from these steers was then used as source of inoculum, given via direct rumen injection, to ~10% of the remaining herd at Lansdown, spreading 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 successful transfer of protection.

In 1995, production of the inoculum in an *in vitro* fermenter began, using rumen fluid containing the mixed bacterial inoculum sourced from cattle at Brian Pastures (Klieve *et al.* 2002). Whilst 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. It was thus postulated that the continually cultured oral inoculum may have lost some strains, undergone genetic modification and/or that it contained other strains with an altered DHP degrading potential (Graham *et al.* 2013; Halliday *et al.* 2013a).

In Indonesia, animals identified as having *S. jonesii* by PCR analysis and confirmed by very low urinary DHP

excretions, were selected as inoculum donors for direct rumen fluid transfer. To test the efficacy of transfer, animals known to be excreting high levels of DHP were inoculated with rumen fluid, which had been maintained in a strict anaerobic state at an appropriate temperature. However, the recipient goats continued excreting 2,3-DHP at levels estimated >1000 mg/L based on colorimetric assay 10 days after inoculation indicating a lack of rapid successful response to inoculation. This occurred during August 2012 and was repeated in November 2012 with cattle and goats. Donors were established as high degraders of DHP and the presence of *S. jonesii* was confirmed by PCR analysis. Again, the ability to degrade DHP (or increased degradation) was not transferred within 10 days. These recent findings are in stark contrast with earlier work where rumen fluid transfer resulted in complete degradation of DHP within 5 days in Indonesia (Jones and Lowry 1984) and within 3 days in Thailand (Palmer *et al.* 2010), and raise questions regarding the ability of *S. jonesii*, and therefore the capacity to degrade DHP, to be easily transferred.

Other microbial control options

Other microbial solutions apart from *S. jonesii* have been investigated as alternate biological control methods. Domínguez-Bello and Stewart (1991) reported the isolation of a DHP degrading *Clostridium* bacteria, which was subsequently lost. 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 obligatory 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 an aero-tolerant gram-negative coccobacillus was isolated, belonging to the genus *Klebsiella*. Whilst 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 able to be incorporated into the production *in vitro* *S. jonesii* inoculum in the future, preventing loss of bacterial numbers in transit. However, further research is required.

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 a glucose molecule 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. Also, by attaching a sugar molecule or metal ion at the active binding hydroxyl site, its toxic chelating ability is reduced (Lowry *et al.* 1985). There is other evidence that the toxic effects of DHP are reduced due to conjugation; recent tests on highly productive animals consuming high leucaena diets in eastern Indonesia showed that they were excreting high levels (estimated at > 1000 mg/L using colorimetric analysis) of conjugated DHP. These animals may have developed a coping mechanism of being able to conjugate DHP, without ruminal degradation, thus reducing its toxicity. This may explain the absence of symptoms.

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.* 1964a; Hegarty *et al.* 1976). 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 T₄ 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 is also suggested as a method of detoxification (Hashiguchi and Takahashi 1977). Supplemental mineral 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 (Puchala *et al.* 1995), suggesting that kidney clearance of DHP is more efficient when chelated or conjugated (Puchala *et al.* 1995). Whilst 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 (Puchala *et al.* 1996).

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 diet. As mimosine toxicity rarely presents in animals regularly consuming leucaena and since DHP toxicity is a factor of both time on leucaena and amount of leucaena in diet (Hammond 1995), moderate levels of leucaena in diet for short periods (\leq 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 other feed sources can reduce the toxic effects associated with DHP. However, reducing consumption of leucaena limits the LWG potential of a fattening system. Given that leucaena is often one of the only feedstuffs available during the extended dry season in many tropical smallholder feeding systems, an effective biological control mechanism is the most practical solution to leucaena toxicity.

An example of limiting exposure is found in fattening systems in Sumbawa, eastern Indonesia, many of which feed high levels of leucaena to Bali bulls (up to 100% of diet), and typically fatten over a 4-6 month period (Panjaitan *et al.* 2013). Farmers report that initial hair loss is common (probably due to mimosine toxicity) followed by complete recovery. They also report that an initial aversion of naïve animals to high leucaena diets can be overcome by using a mixture of fresh faeces from an older bull and fresh leucaena leaves as a source of 'inoculum' (Panjaitan *et al.* 2013).

Other less effective management strategies include drying leucaena. While this converts most of the mimosine to DHP (Hegarty *et al.* 1964b; Wee and Wang 1987), it does not overcome the toxic effects of DHP itself.

Future research, development and extension directions

Leucaena toxicity, as indicated by high levels of urinary DHP, is still common through tropical countries where leucaena is fed to ruminants.

Farmers and Government agencies promoting the use of leucaena are often ignorant of the effects of leucaena toxicity, especially since there are usually no clinical symptoms. It is vital that research and education are continued to improve understanding and management of this extremely productive legume, in order to optimize animal production and eliminate negative impacts on animal health.

Bacterial control offers the most beneficial and practical solution, provided easy transfer mechanisms are developed, and long-term persistence of efficacy of DHP degradation can be assured. Future research goals include: (1) improved molecular methods for the identification and understanding of the functionality of *S. jonesii* and the *Synergistes* phylum; (2) alternate microbial control options; (3) improved inoculation protocols within Australia and developing countries; and (4) alternate methods of detoxifying DHP.

Acknowledgements

The following are acknowledged for their contributions: Dr Tanda Panjaitan, Dr Dahlanuddin, Dr Jacob Nulik, and Debora Kana Hau for experimental support in Indonesia; the ACIAR supported Indonesian field researchers for their physical assistance with collection of rumen fluid and urine samples; and Dr Scott Dalzell and Hayley Giles for their guidance. The work was done with the financial support of ACIAR.

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