CSIRO PUBLISHING

Animal Production Science, 2018, **58**, 568–576
http://dx.doi.org/10.1071/AN16394

Accumulation and depletion of indospicine in calves (*Bos taurus*) fed creeping indigo (*Indigofera spicata*)

Mary T. Fletcher[A,B,D], *Keith G. Reichmann*[B], *Selina M. Ossedryver*[B], *Ross A. McKenzie*[B], *Phillip D. Carter*[C] *and Barry J. Blaney*[B]

[A]Centre for Animal Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Health and Food Sciences Precinct, 39 Kessels Road, Coopers Plains, Qld 4108, Australia.
[B]Biosecurity Queensland, Animal Research Institute, Yeerongpilly, Qld 4105, Australia.
[C]Biosecurity Queensland, Tick Fever Centre, Wacol, Qld 4076, Australia.
[D]Corresponding author. Email: mary.fletcher@uq.edu.au

Abstract. Prolonged consumption of *Indigofera* pasture plants can cause both hepatotoxicosis and reproductive losses in grazing animals with the responsible toxin indospicine forming persistent tissue residues. Separate accumulation and depletion feeding trials were undertaken in calves fed *Indigofera spicata* (3 mg indospicine/kg bodyweight) to ascertain the appearance and elimination of indospicine from various tissues. In the accumulation trial indospicine concentrations increased throughout the 42-day feeding period with maximum levels of 15 mg/L in plasma and 19 and 33 mg/kg in liver and muscle, respectively. In the depletion trial, calves were fed *I. spicata* for 35 days, after which the plant was withdrawn from the diet. The rate of elimination was relatively slow with estimates of half-life being 31, 25 and 20 days for muscle, liver and plasma, respectively. Indospicine levels measured in bovine tissues in this trial are comparable with levels in horsemeat and camel meat reported to cause fatal hepatoxicity in dogs, a species known to be susceptible to this toxin. The persistence of indospicine residues in bovine tissues and the widespread distribution of *Indigofera* species in tropical and sub-tropical grazing lands warrant further investigation, as indospicine has been established as causing reproductive losses and likely contributes to calf losses in these regions.

Additional keywords: carcass residues, cattle, *Indigofera linnaei*.

Received 17 June 2016, accepted 20 September 2016, published online 28 November 2016

Introduction

Pasture plants contain a range of toxins that can poison grazing ruminants (van Raamsdonk *et al*. 2015). Such chemicals, if residual in animal tissues, may also impact further along the food chain (Edgar *et al*. 2011; Fletcher *et al*. 2011*a*, 2011*b*; Dorne and Fink-Gremmels 2013). In Australia several reviews have documented the range of toxins that could affect grazing animals and prioritised the risk that these pose to both health and trade (Sykes *et al*. 1997; SCARM 2000). However, such assessments are compromised by the limited knowledge of the range of plants and toxins, their concentrations and toxicity data, particularly for those in the extensive pastoral areas of northern Australia.

Plants of the *Indigofera* genus are one such example. There are ~700 *Indigofera* species in tropical and warm temperate regions of the world (Mabberley 1997), with ~30 *Indigofera* species present in Australia (Wilson and Rowe 2004; Wilson and Rowe 2008*a*, 2008*b*). Of these *Indigofera linnaei* and *I. spicata* have been associated with both animal poisonings and also the formation of tissue residues with the potential to cause secondary poisonings of dogs (Hegarty *et al*. 1988; FitzGerald *et al*. 2011; Ossedryver *et al*. 2013). *Indigofera spicata* originates in tropical Asia and Africa and is now common in Australia following introduction as a pasture legume (Cook *et al*. 2005). *I. linnaei* is native to Australia but also extends from India through Malaysia and Indonesia (Wilson and Rowe 2008*a*). Their attributes include high protein concentrations and tolerance to drought and salinity and consequently have been investigated as potential pasture and feed supplements. *I. spicata* and the closely related *I. hendecaphylla*, in particular, have been grown as a pasture legume in many tropical countries including the USA and Australia (Ossedryver *et al*. 2013; Fletcher *et al*. 2015). However, these taxa contain the hepatotoxin indospicine (Fig. 1), that has been associated with several field reports of poisoning in ruminants including reproductive losses (Fletcher *et al*. 2015).

Indospicine (2(*S*)-2,7-diamino-7-iminoheptanoic acid, previously also described as L-2-amino-6-amidinohexanoic acid) is a non-proteinogenic amino acid first isolated and characterised by Hegarty and Pound from *I. spicata* (Hegarty and Pound 1968, 1970). It is an arginine analogue, which interferes with hepatic protein synthesis and leads to chronic liver damage in laboratory and domestic animals, and

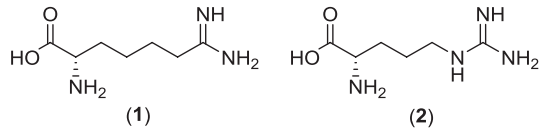

Fig. 1. Structure of *Indigofera* toxin, indospicine (1) and arginine (2).

reproductive losses in livestock (Fletcher *et al*. 2015). Field reports and feeding trials have shown the plant to be heptatotoxic to cattle, sheep, rabbits, mice and rats (Nordfeldt *et al*. 1952; Hutton *et al*. 1958*a*, 1958*b*; Christie *et al*. 1975) with indospicine identified as the responsible toxin. Indospicine was also identified as the teratogen responsible for cleft palate in rats as well as fetal loss (Pearn and Hegarty 1970). Interestingly, horses do not develop liver disease, even though high concentrations of indospicine accumulate in their liver and muscle (up to 30 mg/kg), but dogs fed meat from such horses accumulate indospicine in similar tissues and develop severe chronic liver damage (Hegarty *et al*. 1988). 'Birdsville horse disease', in horses grazing *I. linnaei*, is a neurological condition attributed to another toxin, 3-nitropropionic acid, glycosides of which are present in *I. linnaei* (Majak 1992). An analogous syndrome occurs in horses consuming *I. spicata* and also *I. lespedeziodes*, which also contain the neurotoxin 3-nitropropionic acid, as well as indospicine (Lima *et al*. 2012; Ossedryver *et al*. 2013). In ruminants 3-nitropropionic acid can be detoxified by ruminal bacteria (Majak and Pass 1989), explaining the absence of reports of an equivalent neurological condition in cattle.

Concern for the proven liver toxicity of horsemeat for dogs had regulatory repercussions, with the Northern Territory Meat Industries Act (NTDPIF 2010) banning slaughter of horses, donkeys, mules or hinnies for human consumption if the animal exhibits signs of being affected by Birdsville horse disease (*I. linnaei* poisoning) or for pet food if it has been in an area in which Birdsville horse disease occurs (south-west Queensland and central Northern Territory). Since then canine fatalities have also resulted from the consumption of indospicine-contaminated camel meat from central Australia (FitzGerald *et al*. 2011), and this has increased the interest in indospicine residues in other animals grazing similar areas in which *Indigofera* is present.

Although indospicine is toxic in several animal species, there have been no reports of human illness associated with *Indigofera* spp., or with meat or offal from livestock with access to this plant. However, given that indospicine-containing plants are widely consumed by livestock throughout the world, it is important to ascertain basic toxicological information on indospicine, including its accumulation in and elimination from tissues following feeding. Such information is also of importance in arid regions of Australia, where *Indigofera* is abundant (Wilson and Rowe 2008*a*) and likely contributes to known high pre- and peri-natal reproductive losses in cattle in these regions (Fletcher *et al*. 2015).

Materials and methods

Indospicine standard

Indospicine standard was kindly provided by Sandra Pollitt (The University of Queensland, Brisbane).

Plant material

Indigofera spicata (creeping indigo) was sourced from two locations in the Brisbane metropolitan area, namely from grassy areas besides Schultz's canal (Queensland Herbarium voucher AQ751279) and from grassy paddocks at the Animal Research Institute (ARI), Yeerongpilly (AQ751378). Plant, ~126 kg for each trial, was collected for the initial accumulation feeding trial and for the depletion trial, with the plant contribution from the ARI site to the total, being 44% and 34%, respectively. Plant material, including flowers and seed pods, was collected with the bulk plant sample stored at −20°C. Just before each trial, it was semi-thawed, chaffed, mixed, sampled for analysis and refrozen in smaller quantities for feeding.

Animals and sampling

These feeding trials were conducted taking full account of the welfare of the calves under DPI&F Animal Ethics Committee Approval ARI 12/04/2005 and SA 2006/06/124. For each trial, three 6-month-old *Bos taurus* calves (mixed breed), weaned, and ruminating were housed in individual enclosed cattle pens (4 m × 3 m). Feed bins, placed internally on railings, enabled careful monitoring of plant intake and animals had *ad libitum* access to water via an automatic drinker. Calves were treated for gastrointestinal nematode infestations with broad-spectrum wormer before a 4-week adaption period in their pens. Two weeks before the start of the trial, jugular blood samples (3 × 10 mL) were taken along with liver and muscle biopsies from each calf. Approximately 250 mg was collected from the liver using an aspiration technique with a cannula and trochar (Dick 1952), while excision from the rump muscle as previously described (Fletcher *et al*. 2011*b*) routinely provided 500 mg of muscle tissue. Samples were frozen immediately in liquid nitrogen and stored at −40°C before assay. Blood was collected for haematology into EDTA anticoagulent and for clinical biochemistry and toxin assay into lithium heparin. Plasma separated after centrifuging at 3200*g* (ambient temperature) was stored at −40°C before indospicine assay. Haematological values (haemoglobin concentration, packed cell volume, erythrocyte, platelet, total and differential leucocyte counts and erythrocyte indices) were measured by ABX VET ABC Hematology Analyser (ABX Diagnostics, Montpellier, France). Chemical concentrations and enzyme activities (aspartate aminotransferase, creatine kinase, glutamate dehydrogenase, γ glutamyl transferase, total protein, albumin, globulin, calcium, magnesium, total and conjugated bilirubin, urea and creatinine) were measured in plasma using an Olympus AU400 (Olympus, Tokyo, Japan). Before necropsy animals were killed humanely by captive bolt pistol followed immediately by severing the spinal cord at the atlanto-occipital joint. Samples of liver, kidney and heart and skeletal muscle were frozen for indospicine assay and a complete range of tissues collected into 10% buffered neutral formalin for histopathology.

Husbandry and management

During the pre-feeding phase calves were fed a base diet of chaffed Rhodes grass (*Chloris gayana*) and lucerne (*Medicago sativa*) with a small quantity of diluted molasses (~50 mL). The molasses was included in case it was necessary to overcome

palatability problems after *Indigofera* was introduced. During each day of the *Indigofera* feeding phase, the allocated amount of *Indigofera* was mixed with the basic lucerne/grass chaff (~2.5 kg for a 130-kg calf) such that animals were fed at maintenance (2% of bodyweight (BW) as dry matter). The introduction of *Indigofera* was phased in over the first 5 days to address possible palatability problems, however the animals readily accepted the added *Indigofera* and molasses was not required. Thereafter *Indigofera* was fed at the full daily dose (1 kg for a 130-kg calf). To achieve this, frozen *Indigofera* was semi-thawed and amounts to be fed for the subsequent 2–3 days pre-weighed and stored at 4°C. Animals were fed once daily at 8am and feed intake monitored by weighing feed residue. The health of the animals was monitored daily throughout the trial, with clinical signs and rectal temperature and heart and respiratory rates recorded. Animals were weighed weekly, with this BW being used to calculate the amount of *Indigofera* fed for the subsequent 7 days.

Indospicine assay

Indospicine was extracted from plasma and tissues by homogenisation in 0.05% trifluoroacetic acid (40 µL plasma/mL and 20 mg tissues/mL) followed by centrifugal ultrafiltration (10 000*g*) to physically deproteinise the extract (Millipore Microcon YM-3 centrifugal filter unit). Liquid chromatography (LC) separations were performed using a Waters 3-µm Atlantis dC_{18} column (4.6 × 150 mm) at 30°C with a flow rate of 0.4 mL/min and an injection volume of 20 µL. The mobile phase was a mixture of (A) 0.05% trifluoroacetic acid and (B) acetontrile with a gradient as follows: 0–4 min, 100% A; 4–5 min, 100% A–70% A; 5 –17 min, 70% A; 17.01–25 min, 100% A. Mass spectrometry (MS) detection was performed using a Quattro Premier Micromass triple quadrupole mass spectrometer, equipped with an electrospray ionisation (ESI) probe used in positive mode. The mass spectrometer response was tuned to a solution of indospicine. The capillary voltage was 3.0 kV; the desolvation and cone gases of nitrogen flow were set at 645 L/h and 47 L/h respectively. The desolvation and source temperature was set at 350°C and 150°C respectively. Argon was used as collision gas for tandem mass spectrometry (MS/MS) with a flow rate of 0.3 mL/min, and the collision energy was set at 21 eV and cone voltage at 25 V. Indospicine quantitation was performed based on the 174.03 >111.05 single reaction monitoring (SRM) transition, with a secondary SRM 174.03 >84.10 as confirmation. Indospicine concentrations were quantitated against indospicine standard solutions prepared in 0.05% trifluoroacetic acid (0.002–4.32 mg/L indospicine).

Representative plant samples were dried, milled and similarly extracted in 0.05% trifluoroacetic acid (25 mg dried plant/mL) followed by dilution (1 : 20) in trifluroacetic acid before ultrafiltration. Due to high concentrations of indospicine, plant extracts were further diluted 1 : 4 before LCMS analysis.

Indigofera spicata – *accumulation feeding trial*

Following the adaption period, three calves (A1, A2 and A3) of 110–130 kg initial BW, were fed *I. spicata* daily for 42 days at a rate to supply 3 mg indospicine/kg BW.day. Daily dose was calculated on the basis that the collected *Indigofera spicata* has been estimated to contain 0.38 mg indospicine/g wet weight (1.1 mg/g dry matter) and animals received a daily dose of ~1 kg of plant per animal (based on BW of 130 kg) per day or ~3 mg indospicine/kg BW.day. Jugular blood was collected 14 days before commencement of feeding, at the commencement of feeding and every 6–7 days thereafter for clinical biochemistry and indospicine assays and every 14–21 days for haematology. Liver and skeletal muscle biopsies were collected weekly from alternate animals. Necropsy was carried out on Day 42 of feeding. At necropsy calves weighed 140–155 kg.

Indigofera spicata – *depletion feeding trial*

Feeding of *Indigofera* was undertaken as in the previous trial, with the same amount of *Indigofera* fed to three calves (D1, D2 and D3) of 120–130 kg initial BW, on a daily basis again delivering 3 mg indospicine/kg BW.day. Feeding of this plant was discontinued at Day 35, after regular biopsies had shown that tissue concentrations had plateaued. Continuation of the trial then involved monitoring for 7 months after cessation of *Indigofera* intake. The calves were moved to different individual pens and fed a complete balanced cattle diet in pelleted form for this phase.

Based on data from the preceding accumulation trial, liver and muscle biopsies were taken from all calves on Days 23, 29 (muscle only) and 34 of plant feeding. Likewise, biopsies of both tissues were collected on Days 10, 34, 65, 98, 129 and 164 of the depletion phase (44, 68, 99, 132, 163 and 198 days from commencement of feeding) and muscle only on Days 3, 6, 17, 24, 49, 83, 114 and 192 of depletion (37, 40, 51, 58, 83, 117, 148 and 226 days from commencement of feeding). Blood for clinical biochemistry and indospicine assays was taken every 3–5 days during feeding and the commencement of the depletion phase but less regularly (between 5 and 15 days) thereafter, whereas blood for haematology was taken on approximately every third blood sampling occasion. Necropsy was undertaken on Day 217 of depletion (251 days from commencement of feeding). During the depletion phase animals were monitored daily (feed and water intake, and general health), but temperature, heart and respiratory rates and BW were recorded at greater intervals than previously. At necropsy calves weighed 206–230 kg.

Half-life determination

For the calculation of half-lives for the depletion of indospicine from tissues, several nonlinear functional forms were trialled, with exponential decay appearing the most appropriate on the basis of fit and biological understanding. The lower asymptote was not significantly ($P > 0.05$) different from zero, so it was omitted from the model. For the liver and plasma data, significant ($P < 0.05$) differences were found between the animals. These were shown to be due to differences in the intercept constants, which indicate different 'initial loadings' for these animals. In none of the three tissue samples (liver, muscle or plasma) were significant differences found between the rates of decay for the different animals, so a single rate parameter was adopted within each dataset. All models were fitted using Genstat (2007).

Results

Indospicine analyses

In the LC-MS/MS, indospicine produces a single charged molecular ion $(M+H)^+$ 174 under ESI+ conditions, and quantitation was performed based on the 174→111 SRM transition, which is unique to indospicine and provides a highly specific detection method. This LC-MS/MS method demonstrates good linearity over a wide range of concentration from 0.002 to 4.32 mg/L in solution (corresponding to 0.25–108 mg/L in plasma and 0.50–216 mg/kg in meat tissue) with correlation coefficients of 0.995 or greater. Results are highly reproducible from tissue samples spiked at 0, 2.5, 5 and 50 mg/kg with average RSD 7.6% and recoveries in the range 65–114% depending on the tissue, with average recoveries for five replicates at each concentration for muscle (114%) and heart (100%) being higher than that for plasma (69%), kidney (65%) and liver (63%). Deviations from 100% were largely attributed to matrix effect. The reproducibility of the indospicine analysis is seen in the low standard deviations obtained in replicate analysis of all necropsy tissues (Tables 1 and 2). The limit of quantitation (LOQ) for indospicine by this LC-MS/MS method was 0.05 mg/L in plasma and 0.1 mg/kg in tissue samples. This limit of detection is significantly lower than the 2 mg/kg limit of detection in horsemeat previously reported by high performance liquid chromatography and ultraviolet detection (HPLC-UV) with phenylisothiocyanate derivitisation (Pollitt *et al*. 1999; Pollitt 2001), and the specificity of the SRM transitions also provides a greater confidence in the analysis of these low indospicine concentrations, particularly in the presence of other amino acids.

Analysis of indospicine in feeding trial plant material by LC-MS/MS gave comparable analysis results to that obtained by the Waters AccQ•Tag amino acid analysis of the same samples (within 10%). Spiked addition of indospicine to *Indigofera* plant extracts at levels equivalent to 0.74, 1.5 and 2.2 mg/kg showed no apparent matrix effect in LC-MS/MS analysis with average recoveries of 104%.

Table 1. Indospicine concentration of tissue replicates at necropsy from *I. spicata* accumulation feeding trial

Tissue	*n*	Indospicine (s.d.) (mg/kg)		
		Calf A1	Calf A2	Calf A3
Muscle	6	13.3 (1.5)	17.5 (3.3)	21.3 (2.6)
Heart	3	19.4 (1.5)	23.9 (1.9)	17.9 (4.5)
Kidney	3	13.6 (0.6)	12.8 (0.8)	12.2 (0.5)
Liver	3	9.3 (0.7)	15.1 (2.8)	12.7 (0.9)

Table 2. Indospicine concentration of tissue replicates at end of *I. spicata* depletion feeding trial

Tissue	*n*	Indospicine (s.d.) (mg/kg)		
		Calf D1	Calf D2	Calf D3
Muscle	2	< LOQ[A]	< LOQ	< LOQ
Heart	2	0.10 (0.01)	0.15 (0.02)	Trace
Kidney	2	< LOQ	< LOQ	< LOQ
Liver	5	0.17 (0.06)	0.18 (0.05)	0.15 (0.04)

[A]Detectable indospicine but below LOQ 0.1 mg/kg.

Indigofera spicata – *accumulation feeding trial*

All three calves remained clinically healthy throughout the trial with average BW increasing from 122 kg on Day 1 to 148 kg on Day 43. Two calves had mild diarrhoea over a couple of days and one had an elevated temperature on one day resulting in the administration of an antibiotic. Both conditions resolved quickly. The *Indigofera* plant material was quite palatable, with calves consuming all the plant, generally within a short period of time after feeding. The quantity of *Indigofera* fed daily varied from 880 g up to 1.15 kg as a result of increasing BW.

Intake of this plant produced no clinical signs or significant variation in biochemical or haematological values in any of the animals during the entire trial. Likewise, no lesions were seen at necropsy nor were any significant histopathological changes seen in any tissues collected at necropsy. Concentrations of indospicine in plasma, liver and muscle increased over the feeding period reaching maximums of 15 mg/L in plasma and 19 and 33 mg/kg in liver and muscle, respectively (Fig. 2). Analysis of necropsy muscle, liver, heart and kidney samples demonstrated relatively similar indospicine concentrations in all tissues with high reproducibility between replicate samples (Table 1).

Indigofera spicata – *depletion feeding trial*

All the animals remained healthy, with average BW increasing from 122 kg at Day 1 to 135 kg on the final plant feeding day (Day 35) and 217 kg at necropsy (Day 217 of depletion). This intake produced no clinical signs of disease, no significant necropsy lesions or histopathological changes, or variations in biochemical or haematological values in any of the animals over the feeding and depletion phases of the trial.

Feeding of *Indigofera* was discontinued at Day 35, after regular biopsies had shown that tissue indospicine concentrations had plateaued. Subsequent depletion was monitored regularly, with concentrations of indospicine dropping slowly in plasma, liver and muscle (Fig. 3). Depletion was terminated at Day 251 (217 days after cessation of *Indigofera* feeding) when indospicine concentrations were approaching or below the LOQ for indospicine. Residual concentrations of indospicine at necropsy are shown in Table 2. The calculated half-life of elimination for indospicine in plasma, liver and muscle during this depletion phase were 19.8 (confidence interval (CI)18.0–22.0), 25.1 (CI 18.1–40.7) and 31.0 (CI 23.9–44.0) days, respectively. Calculated half-lives for plasma and muscle were significantly different ($P < 0.01$), but neither is significantly different from liver. The exponential decay curves for plasma for each of calves D1, D2 and D3 are shown in Fig. 4. Indospicine plasma concentrations measured in all three calves D1, D2 and D3 correlated reasonably well with both muscle and liver indospicine concentrations during both accumulation and elimination phases of the depletion feeding trial with R^2 of 0.88 and 0.82 respectively (Fig. 5).

Discussion

The absence of any adverse effects on the calves fed ~25% inclusion levels of *Indigofera spicata* in their diet for up to

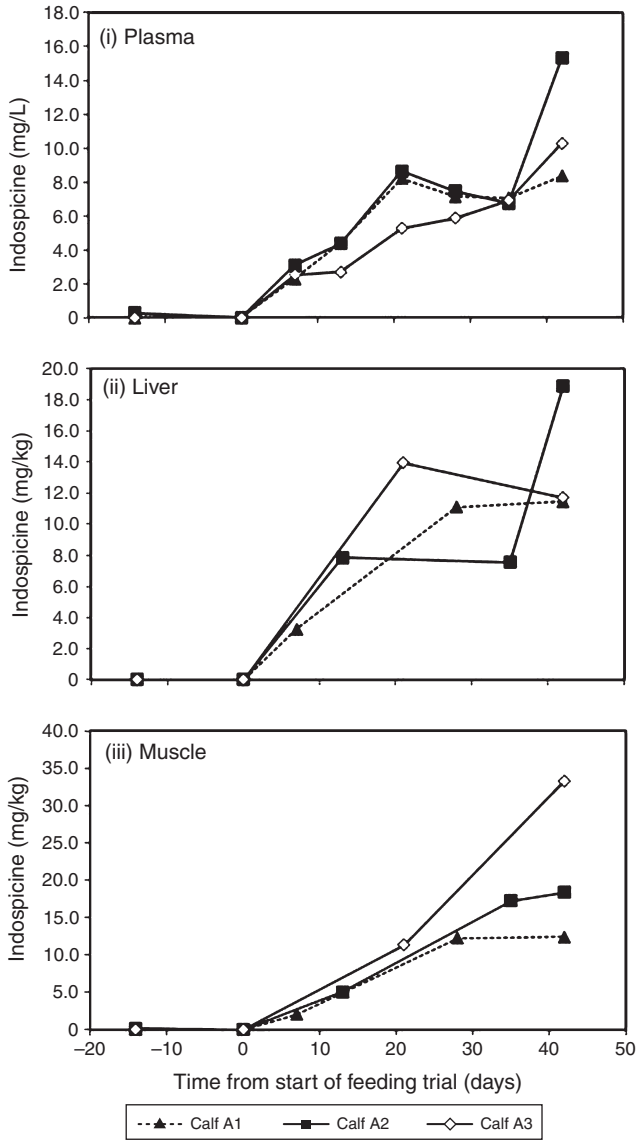

Fig. 2. Indospicine accumulation in (i) plasma, (ii) liver and (iii) muscle tissues of calves A1, A2 and A3 during the 42-day *Indigofera* accumulation feeding trial.

42 days in these trials is comparable with findings by Nordfeldt (Nordfeldt *et al*. 1952) that indicate that much greater doses of this plant and feeding over longer periods are required to produce clinical effects in cattle. In these previous field studies, *I. spicata* intake increasing from 16% to 52% of the diet for up to 44 days caused reproductive toxicosis in cattle, and non-bred heifers fed increasing percentages from 30% to 100% fresh *I. spicata* for 15 days experienced gradual loss of appetite and lost weight (Nordfeldt *et al*. 1952). However, these comparisons typically suffer from a lack of data on plant indospicine concentrations and actual dose rates. In the trials reported here the plant contained 1.1 mg indospicine/g dry weight but, in contrast to other pen trials, the plant was not fed fresh but was collected, mixed for consistency and stored frozen before feeding with a dietary inclusion of ~25% *I. spicata*.

As demonstrated by our trial, the *I. spicata* plant material was a reasonable source of nutrition and the feed intake observations clearly indicate that the plant was quite palatable. It was these same factors that encouraged the use of *I. spicata*, and the closely related *I. hendacaphylla*, as pasture legumes in many tropical countries, until subsequent research highlighted animal health risks due to the presence of the toxin, indospicine (Aylward *et al*. 1987; Abdullah and Rajion 1997). These species nevertheless remain widely consumed by livestock, either intentionally or inadvertently, with native distribution across tropical regions of Africa and Asia, and naturalised status in Australia, Florida and Hawaii (Fletcher *et al*. 2015). The presence of indospicine in pasture plants consumed by livestock is moreover not limited to the *I. spicata*/*I. hendaphylla* complex, with both *I. linnaei* and *I. lespedezioides* known to contain comparable levels of indospicine (Lima *et al*. 2012; Fletcher *et al*. 2014). *I. linnaei* is native across northern Australia, India, south-eastern Asia, and Melanesia (GRIN 2015*a*), and features a large taproot that enables it to withstand dry conditions and respond quickly to rainfall (Gracie *et al*. 2010). In arid central Australia this species can at times form a large proportion of pasture when small rainfall events favour the growth of this species but are insufficient to support the growth of other pasture plants (Gracie *et al*. 2010). Similarly *I. lespedezioides* is native to Mexico and South America (GRIN 2015*b*), and in Brazil is noted to be a predominant feature of some pastures at the end of the dry season (Lima *et al*. 2012). Given the reported palatability of these species, it can therefore be concluded that the intake of indospicine could be significant in cattle grazing any areas where *I. spicata*, *I. hendecaphylla*, *I. linnaei*, or *I. lespedeziodes* occur as a significant proportion of pasture.

Our feeding trials, based on regular biopsy sampling of liver and muscle and of other tissues at necropsy, demonstrated that calves consuming *I. spicata* can accumulate much of the dose as indospicine residues in tissues with no observable ill effect. In the accumulation trial, for example calves fed at 3 mg/kg BW daily for 42 days (estimated total indospicine dose 17.0 g per calf) accumulated average levels of 17.4 mg/kg in muscle or ~1.3 g per calf assuming 50% muscle in calves of average 148 kg BW. The highest concentrations of indospicine were achieved in the initial accumulation feeding trial (calves A1, A2 and A3), despite the quantity of *Indigofera* fed and indospicine content being similar in the subsequent depletion trial (calves D1, D2 and D3). This variation may have been a consequence of limited numbers of experimental animals and considerable animal variation (both between and within animals); for example, indospicine concentrations in muscle from the three calves A1, A2 and A3 ranged from 13 to 21 mg/kg (Table 1) and separate 'biopsy' samples taken at necropsy ranges from 12 to 33 mg/kg for the same animals (Fig. 2). Reports of a previous undocumented feeding trial indicate that a single steer fed *I. linnaei* at 2 kg dry matter per day (percentage intake and BW not recorded) for 6 weeks resulted in indospicine serum concentrations of 3.9 mg/L together with muscle and liver concentrations of 18 and 23 mg/kg respectively (AZRI 1989). Blood indospicine levels in a second animal fed a similar regime was reported to rise from 0.2 mg/L to only 2.2 mg/L and returned to 0.2 mg/L over a period of 3 months. Although not stated, animals used in this study may well have been *Bos indicus* (common in central

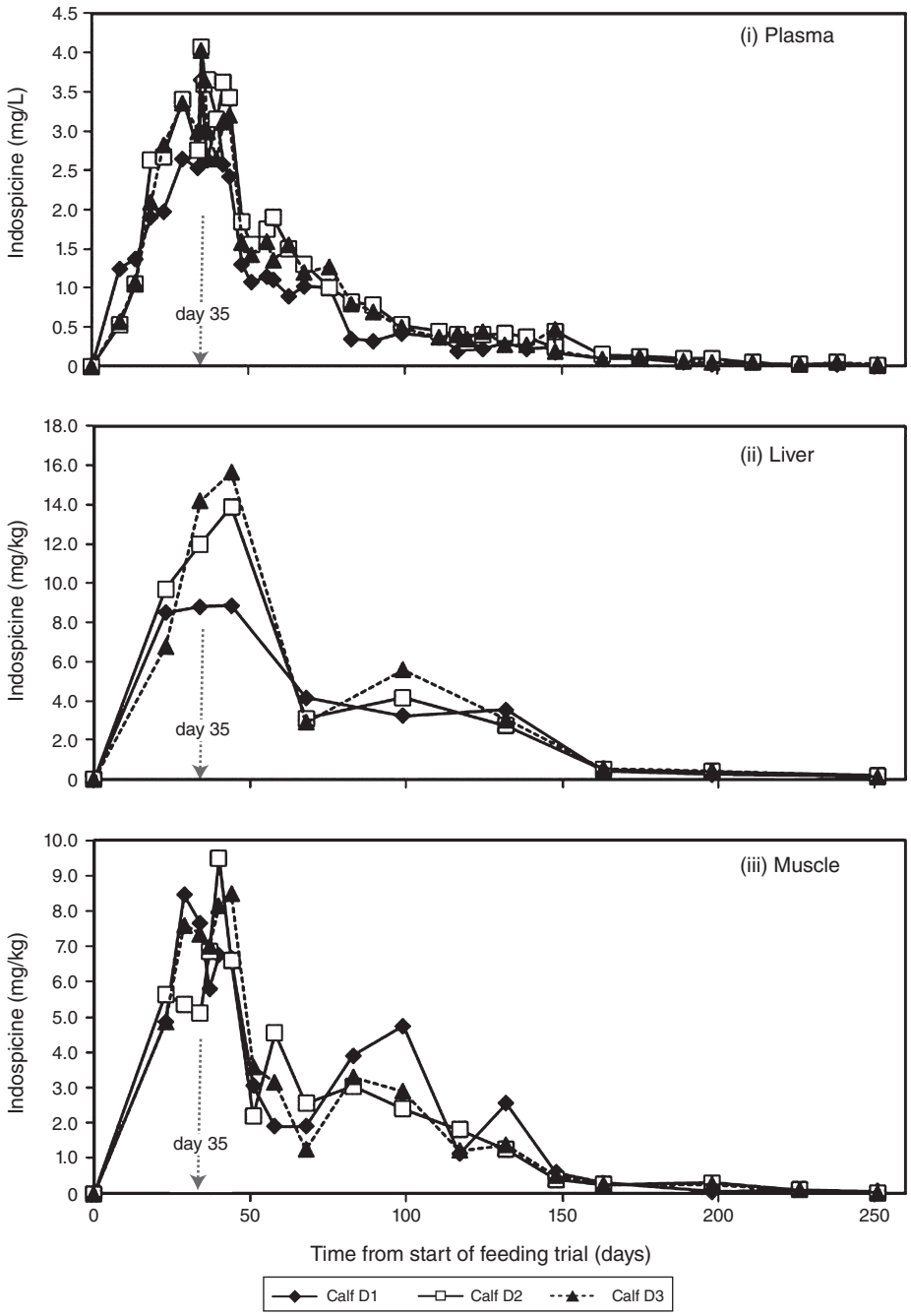

Fig. 3. Indospicine accumulation and depletion in (i) plasma, (ii) liver and (iii) muscle tissues of calves D1, D2 and D3 during and after the 35-day *Indigofera* feeding phase of the depletion trial.

Australia where this trial was conducted) rather than the *Bos taurus* in our study. This variation in indospicine blood levels can again be attributed to individual animal differences, and may in part be influenced by prior exposure to this plant (as evidenced by the measured indospicine blood concentrations at the commencement of the trial).

Indospicine muscle concentrations comparable to our bovine tissues have also been reported in horses, with meat containing 16–30 mg indospicine/kg obtained from horses that had either been fed *I. linnaei* or grazed pastures containing this plant (Hegarty *et al*. 1988). These tissue concentrations were sufficient to induce hepatotoxicosis in dogs fed this horsemeat (Hegarty *et al*. 1988), and would suggest that the bovine meat from our accumulation trial would have similar effects if fed to dogs, a species highly susceptible to this toxin. Pollitt detected concentrations of 12.9 and 21.2 mg/kg in horses grazing *I. linnaei* (Pollitt *et al*. 1999), and 42.1 mg/kg in a horse suffering Birdsville horse disease (Pollitt 2001). In an unpublished report cited by

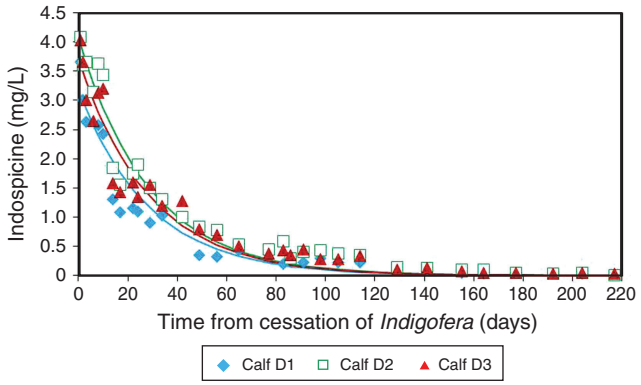

Fig. 4. Exponential decay curves for indospicine in plasma of calves D1, D2 and D3 during the elimination phase of the depletion trial. Equations for fitted curves, (calf D1): Indospicine concentration = $3.19*0.96562^{\text{days depletion}}$; (calf D2): Indospicine concentration = $4.08*0.96562^{\text{days depletion}}$; (calf D3): Indospicine concentration = $3.72*0.96562^{\text{days depletion}}$.

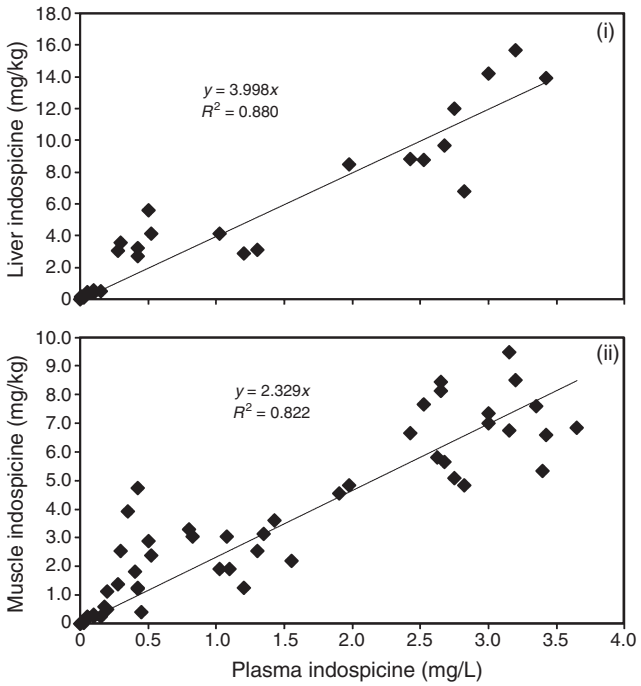

Fig. 5. Correlation of indospicine concentrations in (i) liver and (ii) muscle with corresponding indospicine plasma concentrations measured in all three calves D1, 2 and D3 during both accumulation and elimination phases of the depletion feeding trial.

Pollitt (2001), Hegarty and Simpson apparently reported that the incidence of indospicine contamination of horsemeat in central Australia was widespread and 'indospicine was detected at concentrations ranging from 1 to 7 mg/kg in nearly 50% of samples of horsemeat collected from abattoirs and from the field in areas where *I. linnaei* grows and Birdsville disease occurs.' In all these cases the intake of *Indigofera* (and indospicine) was unknown. Other animal species also accumulate indospicine when administered in the pure form to dogs (Kelly *et al.* 1992; Young 1992) or as *Indigofera* plant material to rabbits (Pollitt 2001) and goats (Young 1992), demonstrating that the absorption and tissue retention of indospicine is similar across species, and that differences in susceptibility to hepatotoxicosis are related to species-specific metabolic processes (with dogs noted to be particularly susceptible).

Secondary poisoning in dogs has previously been confirmed by demonstrating that when indospicine-contaminated meat from horses grazing *I. linnaei* was fed to dogs they developed severe chronic liver damage (Hegarty *et al.* 1988). Dogs fed indospicine-contaminated horsemeat (Hegarty *et al.* 1988) or with pure indospicine added to their diet (Kelly *et al.* 1992) accumulated similar or greater levels of indospicine in these tissues that reflect dose rates and period of feeding. Long-term ingestion (70 days) of indospicine-contaminated horsemeat (~0.23 mg indospicine/kg BW.day) by dogs resulted in tissue concentrations in muscle, liver and pancreas of ~10, 20 and 30 mg/kg respectively (Hegarty *et al.* 1988) whereas 0.8 mg indospicine/kg BW.day for 32 days produced levels 3–5 times higher in muscle and liver tissues (pancreas not reported) (Hegarty *et al.* 1988).

A better understanding of the significance of these tissue indospicine concentrations required evaluation of their persistence in tissues. Our depletion feeding trial monitored tissue concentrations following equilibrium, the point at which there was a broad balance between accumulation and elimination of indospicine in body tissues. This occurred at ~35 days; however, this datum was variable and was based on a small number of animals. In this depletion study indospicine accumulated in plasma, liver and muscle was only slowly excreted. A basic toxicokinetic evaluation showed indospicine concentrations in plasma conformed to a first-order elimination process (Fig. 4), suggesting that indospicine rapidly equilibrates between blood and tissues as explained by the 'one compartment' model (Klaasen 2013) The majority of xenobiotics fall into this category. This rapid equilibration suggests there is little tissue binding of indospicine, a conclusion compatible with the finding that indospicine exists in tissues in the soluble form (Hegarty *et al.* 1988).

Although this model predicts a similar half-life in all tissues, the small number of animals, variable data and the substantially reduced number of sampling points for tissue indospicine concentrations, relative to plasma ($t_{1/2}$ = 20 days), might account for the observed differences of greater half-lives in liver and muscle (25 and 31 days). However, these estimates were not significantly different from that of plasma. Others have also observed this relatively slow elimination of indospicine from other animal species with the toxin detected in the serum from a horse 2 months after consumption of *I. linnaei* (Hegarty 1992), and in a goat 3 months after cessation of *I. linnaei* feeding (Young 1992), and in liver and muscle of a dog 55 days after cessation of parenteral dosing of indospicine (Young 1992). Pollitt showed in rabbits fed *I. spicata* seed that indospicine concentrations in liver were generally higher than in muscle and that it was detectable in serum 35 days after cessation of feeding (Pollitt 2001). Our interrogation of the limited data provided by Pollitt in rabbits (Pollitt 2001) and Young in a goat (Young 1992) suggests that indospicine serum/plasma half-life is ~5 and 13 days, in these species, respectively.

Knowledge of the half-life of indospicine in tissues can provide a better understanding of the consequences of

exposure to *Indigofera* including the evaluation of strategies to reduce indospicine residue concentrations in grazing animals and also impacts on animal productivity which can clearly continue well beyond the cessation of *Indigofera* intake. It also helps assess the value of indicators for indospicine consumption, including the use of plasma as an indicator of residual indospicine in meat and liver. Investigation of the correlation of plasma with liver and muscle indospicine concentrations throughout the depletion trial (Fig. 5), established that plasma indospicine does indeed provide predictive information on levels in other tissues, with liver and muscle indospicine concentrations being roughly 4 and 2.3 times, respectively, that measured in plasma at any sampling event. Measurement of tissue indospicine in field animals cannot however establish either the timing or magnitude of *Indigofera* consumption, and whether a measurement of say 2 mg/kg in muscle is indicative of a recent low *Indigofera* intake or of a considerably larger *Indigofera* intake at some time in the past.

Conclusions

These feeding and depletion trials confirm that in cattle, as in other animal species including dogs (Kelly *et al*. 1992; Young 1992), rabbits (Pollitt 2001) and horses (Hegarty *et al*. 1988), indospicine is found in a variety of tissues with a relatively long half-life of elimination, suggesting that biotransformation is relatively slow. Indospicine contains an unusual amidino group and no mammalian enzymes have been reported that are able to degrade such a group. This may explain the persistence of un-metabolised indospicine in animal tissues. Further investigation is required to ascertain impacts of indospicine residues on animal productivity, particularly on reproductive losses, in cattle grazing pastures in areas where *Indigofera* species can be prevalent.

Acknowledgements

The authors are very grateful for funding provided by Meat and Livestock Australia (Project Number AHW.017) to carry out this study The authors gratefully acknowledge the staff of Biosecurity Sciences Laboratory and other staff of the Queensland Department of Agriculture and Fisheries (formerly Department of Primary Industries and Fisheries) for carrying out laboratory testing and with assistance in plant collections. Andrew Kelly and other Animal Research Institute farm staff provided invaluable assistance in daily management of the calves. David Mayer conducted statistical analysis. Plant identifications were determined by staff of Queensland Herbarium (Environmental Protection Agency, Qld).

References

Abdullah AS, Rajion MA (1997) Dietary factors affecting entero-hepatic function of ruminants in the tropics. *Animal Feed Science and Technology* **69**, 79–90. doi:10.1016/S0377-8401(97)81624-0

Aylward JH, Court RD, Haydock KP, Strickland RW, Hegarty MP (1987) *Indigofera* species with agronomic potential in the tropics. Rat toxicity studies. *Australian Journal of Agricultural Research* **38**, 177–186. doi:10.1071/AR9870177

AZRI (1989) 'Indospicine in beef (Arid Zone Research Institute).' Northern Territory Department of Primary Industries and Fisheries Technical Annual Report 1987–1988. pp. 39. (NTDPIF: Alice Springs)

Christie GS, Wilson M, Hegarty MP (1975) Effects on the liver in the rat of ingestion of *Indigofera spicata*, a legume containing an inhibitor of arginine metabolism. *The Journal of Pathology* **117**, 195–205. doi:10.1002/path.1711170402

Cook BG, Pengelly BC, Brown SD, Donnelly JL, Eagles DA, Franco MA, Hanson J, Mullen BF, Partridge IJ, Peters M, Schultze-Kraft R (2005) Tropical forages: an interactive selection tool – *Indigofera spicata*. Available at http://www.tropicalforages.info/key/Forages/Media/Html/Indigofera_spicata.htm [Verified 17 June 2016]

Dick AT (1952) Improved apparatus for aspiration biopsy of the liver in sheep. *Australian Veterinary Journal* **28**, 234–235. doi:10.1111/j.1751-0813.1952.tb13481.x

Dorne JLCM, Fink-Gremmels J (2013) Human and animal health risk assessments of chemicals in the food chain: Comparative aspects and future perspectives. *Toxicology and Applied Pharmacology* **270**, 187–195. doi:10.1016/j.taap.2012.03.013

Edgar JA, Colegate SM, Boppre M, Molyneux RJ (2011) Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment* **28**, 308–324. doi:10.1080/19440049.2010.547520

FitzGerald LM, Fletcher MT, Paul AEH, Mansfield CS, O'Hara AJ (2011) Hepatotoxicosis in dogs consuming a diet of camel meat contaminated with indospicine. *Australian Veterinary Journal* **89**, 95–100. doi:10.1111/j.1751-0813.2010.00684.x

Fletcher MT, McKenzie RA, Reichmann KG, Blaney BJ (2011*a*) Risks from plants containing pyrrolizidine alkaloids for livestock and meat quality in Northern Australia. In 'Poisonings by plants, mycotoxins and related toxins'. (Eds F Riet-Correa, J Pfister, A Schild, T Wierenga) pp. 208–218. (CAB International: Wallingford, UK)

Fletcher MT, Reichmann KG, Brock IJ, McKenzie RA, Blaney BJ (2011*b*) Residue potential of norsesquiterpene glycosides in tissues of cattle fed Austral bracken (*Pteridium esculentum*). *Journal of Agricultural and Food Chemistry* **59**, 8518–8523. doi:10.1021/jf201342t

Fletcher MT, Tan ETT, Silcock RG (2014) *Indigofera linnaei* – a problem legume across northern pastures. *Proceedings of the Australian Society of Animal Production* **30**, 285.

Fletcher MT, Al Jassim RAM, Cawdell-Smith AJ (2015) The occurrence and toxicity of indospicine to grazing animals. *Agriculture* **5**, 427–440. doi:10.3390/agriculture5030427

Genstat (2007) 'Genstat for Windows, release 9.1.' 9th edn.' (VSN International Ltd: Oxford)

Gracie A, Brown A, Saville P (2010) AgNote: Birdsville Disease (Northern Territory Government). Available at www.nt.gov.au/d/Content/File/p/Anim_Dis/633.pdf [Verified 17 June 2016]

GRIN (2015*a*) *Indigofera linnaei* Ali. (USDA Germplasm Resources Information Network) Available at http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?70382 [Verified 17 June 2016]

GRIN (2015*b*) Taxon: *Indigofera lespedezioides* Kunth. (USDA Germplasm Resources Information Network) Available at http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?20025 [Verified 17 June 2016]

Hegarty MP (1992) Non-metallic chemical residues in toxic plants with potential importance to animal and human health. In 'Vet Update '92'. (Ed. HG Osborne) pp. 323–332. (University of Queensland, Continuing Professional Education: Brisbane)

Hegarty MP, Pound AW (1968) Indospicine, a new hepatotoxic amino acid from *Indigofera spicata. Nature* **217**, 354–355. doi:10.1038/217354a0

Hegarty MP, Pound AW (1970) Indospicine, a hepatotoxic amino acid from *Indigofera spicata*: isolation, structure, and biological studies. *Australian Journal of Biological Sciences* **23**, 831–842. doi:10.1071/BI9700831

Hegarty MP, Kelly WR, McEwan D, Williams OJ, Cameron R (1988) Hepatotoxicity to dogs of horse meat contaminated with indospicine. *Australian Veterinary Journal* **65**, 337–340. doi:10.1111/j.1751-0813.1988.tb14259.x

Hutton EM, Windrum GM, Kratzing CC (1958*a*) Studies on the toxicity of *Indigofera endecaphylla*: I. Toxicity for rabbits. *The Journal of Nutrition* **64**, 321–337.

Hutton EM, Windrum GM, Kratzing CC (1958*b*) Studies on the toxicity of *Indigofera endecaphylla*: II. Toxicity for mice. *The Journal of Nutrition* **65**, 429–440.

Kelly WR, Young MP, Hegarty MP, Simpson GD (1992) The hepatotoxicity of indospicine in dogs. In 'Poisonous plants'. (Eds LF James, RF Keeler, EM Bailey, PR Cheeke, MP Hegarty) pp. 126–130. (Iowa State University Press: Ames, IA)

Klaasen CD (Ed.) (2013) 'Casarett and Doull's toxicology: the basic science of poisons.' (McGraw-Hill: New York, NY)

Lima EF, Riet-Correa F, Gardner DR, Barros SS, Medeiros RMT, Soares MP, Riet-Correa G (2012) Poisoning by *Indigofera lespedezioides* in horses. *Toxicon* **60**, 324–328. doi:10.1016/j.toxicon.2012.04.341

Mabberley DJ (1997) 'The plant book. A portable dictionary of the vascular plants.' 2nd edn. (Cambridge University Press: Cambridge)

Majak W (1992) Mammalian metabolism of toxic glycosides from plants. *Journal of Toxicology. Toxin Reviews* **11**, 1–40. doi:10.3109/15569549209016436

Majak W, Pass MA (1989) Aliphatic nitrocompounds. In 'Toxicants of plant origin, Vol II, *Glycosides*'. (Ed. PR Cheeke) pp. 143–159. (CRC Press: Boca Raton, FL)

Nordfeldt S, Henke LA, Morita K, Matsumoto H, Takahash M, Younge OR, Willers EH, Cross RF (1952) Feeding tests with *Indigofera endecaphylla* Jacq. (Creeping indigo) and some observations on its poisonous effects on domestic animals. *Hawaii Agricultural Experiment Station College of Agriculture University of Hawaii Technical Bulletin* **15**, 5–23.

NTDPIF (2010) Northern Territory of Australia Meat Industries Act (Northern Territory Department of Primary Industries, Fisheries and Mines). Available at http://www.austlii.edu.au/au/legis/nt/consol_act/mia184.txt/cgi-bin/download.cgi/download/au/legis/nt/consol_act/mia184.rtf#_Toc263691324 [Verified 17 June 2016]

Ossedryver SM, Baldwin GI, Stone BM, McKenzie RA, Van Eps AW, Fletcher MT (2013) *Indigofera spicata* (creeping indigo) poisoning of horses. *Australian Veterinary Journal* **91**, 143–149. doi:10.1111/avj.12032

Pearn JH, Hegarty MP (1970) Indospicine – the teratogenic factor from *Indigofera spicata* extract causing cleft palate. *British Journal of Experimental Pathology* **51**, 34–36.

Pollitt S (2001) Residue implications of indospicine, a toxic, non-protein amino acid. PhD thesis, Department of Physiology and Pharmacology, University of Queensland, St Lucia, Australia.

Pollitt S, Hegarty MP, Pass MA (1999) Analysis of the amino acid indospicine in biological samples by high performance liquid chromatography. *Natural Toxins* **7**, 233–240. doi:10.1002/1522-7189(199911/12)7:6<233::AID-NT59>3.0.CO;2-3

SCARM (2000) 'Managing the food safety and trade risks associated with natural toxins in agricultural and fisheries products. Report of the SCARM Working Group on Natural Toxins.' (Standing Committee on Agricultural and Resource Management: Canberra)

Sykes, B, Morgan, I, Harrison, M (1997) A review of natural toxins. A report for the Australian Meat Research Corporation, Sydney, Australia.

van Raamsdonk LWD, Ozinga WA, Hoogenboom LAP, Mulder PPJ, Mol JGJ, Groot MJ, van der Fels-Klerx HJ, de Nijs M (2015) Exposure assessment of cattle via roughages to plants producing compounds of concern. *Food Chemistry* **189**, 27–37. doi:10.1016/j.foodchem.2015.02.050

Wilson PG, Rowe R (2004) A revision of the Indigofereae (Fabaceae) in Australia. 1. *Indigastrum* and the simple or unifoliolate species of *Indigofera*. *Telopea* **10**, 651–682.

Wilson PG, Rowe R (2008*a*) A revision of the Indigofereae (Fabaceae) in Australia. 2. *Indigofera* species with trifoliolate and alternately pinnate leaves. *Telopea* **12**, 293–307. doi:10.7751/telopea20085819

Wilson PG, Rowe R (2008*b*) Three new species of *Indigofera* (Fabaceae: Faboideae) from Cape York Peninsula. *Telopea* **12**, 285–292. doi:10.7751/telopea20085818

Young MP (1992) Investigation of the toxicity of horsemeat due to contamination by indospicine. PhD thesis, School of Veterinary Science, University of Queensland, St Lucia, Australia.