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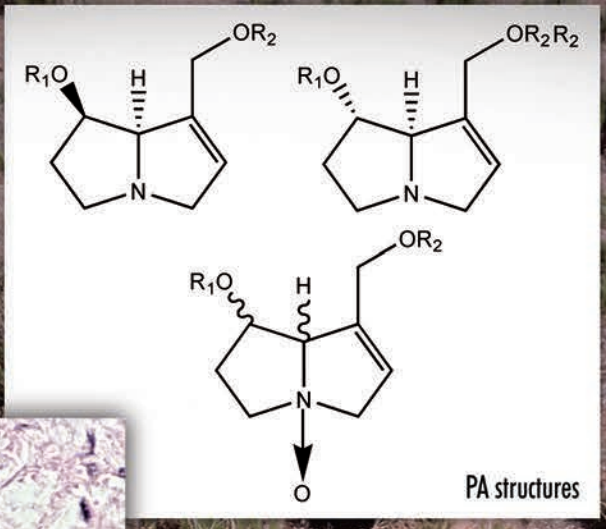
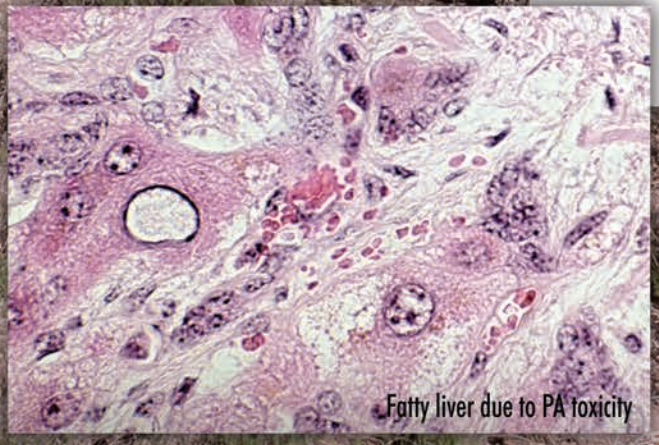
Senecio

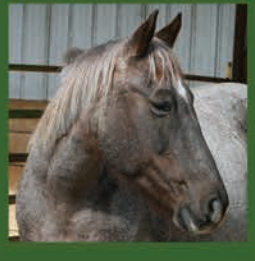


PA structures


Amsinckia


Fatty liver due to PA toxicity

Pyrrolizidine Alkaloids





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Preface

We are pleased to publish this 4th issue of the *International Journal of Poisonous Plant Research* (*IJPPR*). The objectives of the *IJPPR* include providing a forum for publishing original research, case reports, and scientific reviews of poisonous-plant-related investigations or research.

Additionally, we want this journal to provide a central outlet to share new information and technology from around the world on analytical techniques, tools to enhance diagnosis, and methods to reduce or prevent animal poisonings from plants. There is currently no journal, electronic or printed, that specifically focuses on poisonous plant research, and *IJPPR* aims to fill this critical role for scientists, veterinarians, educators, and the general public with an interest in toxic plants and their associated impact on animals and people throughout the world.

We have employed an electronic publication system to make the papers as widely accessible as possible on the ARS website at no charge. It is our intent for *IJPPR* to be an interdisciplinary source bringing together all disciplines with a common interest in poisonous plants.

This issue contains manuscripts that provide diverse research information and photographs of plant poisonings from the United States and other parts of the world. The cover and first three articles represent pyrrolizidine and dehydropyrrolizidine alkaloids and their toxicity to livestock, structural implications on carcinogenicity, and a proposed taxonomic revision for one genus, *Amsinckia*. Also in this issue are reports on lupine toxicity, monofluoroacetate poisoning in sheep, white snakeroot, and rayless goldenrod toxins, and an evaluation of ELISA methods for management and control of annual ryegrass toxicity.

The Editors-in-Chief thank those who have assisted in the production of the 4th *IJPPR* issue, particularly Terrie Wierenga, Editorial Assistant, ARS Poisonous Plant Research Laboratory, Logan, UT; Rosalind James, National Program Leader, ARS Office of National Programs; the staff at the ARS Office of Communications, Beltsville, MD; and others responsible for publishing this online journal. We also thank the *IJPPR* Editorial and Advisory boards for their peer review and recommendations for publication.

Contents

Hydroxylic Solvent-Induced Ring Opening of the Dehydropyrrolizidine Alkaloids Riddelliine and Seneciphylline: Implications for Toxicity and Analytical Studies

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Abstract

Dehydropyrrolizidine alkaloids are potentially hepato-, pneumo-, and geno-toxic plant secondary metabolites that can also cause various cancers in animal models. Riddelliine and seneciphylline are closely related, macrocyclic diester dehydropyrrolizidine alkaloids produced by various species in the Asteraceae. Despite the evidence of carcinogenicity in animal models and the increasing concerns with respect to human health, riddelliine and lasiocarpine are the only dehydropyrrolizidine alkaloids so far officially described as Group 2B carcinogens. This, combined with the ready accessibility of quite pure riddelliine from some plant sources, has led to riddelliine often being used in toxicology studies. A routine HPLC-esi(+)MS analysis of a stored, standard solution of riddelliine in methanol revealed the formation of two new isobaric (*m/z* 382) peaks at slightly longer retention time. A similar formation of two new isobaric (*m/z* 396) peaks was observed for riddelliine in ethanol. The methanol- and ethanol-related compositional changes were also observed with seneciphylline and the *N*-oxides of riddelliine and seneciphylline but not with retrorsine, senecionine, heliotrine, or the *N*-oxides of senecionine and heliotrine. There was no evidence for complete hydrolysis of the alkaloids to the retronecine base. Isolation of the major riddelliine–methanol adduct and subsequent 1D and 2D NMR spectroscopic analysis unambiguously determined that the methanol adds to the riddelliine in a ring-opening transesterification at the allylic C9 ester bond forming a new C7 monoester of retronecine with methylriddelliic acid. The spontaneous formation of riddelliine and seneciphylline adducts should be checked prior to using solutions of riddelliine and seneciphylline, or their *N*-oxides, as analytical standards or for toxicological studies.

Keywords: dehydropyrrolizidine alkaloids, heliotrine, HPLC-esiMS, NMR, *N*-oxides, retrorsine, riddelliine, seneciphylline, senecionine, transesterification

Introduction

The toxicity of the 1-hydroxymethyl-1,2-dehydro-7-hydroxypyrrolizidine alkaloid mono- and diesters (dehydroPAs), produced by numerous plant species in genera of the Boraginaceae, Asteraceae, and Fabaceae, to livestock and to humans is well documented (Bull et al. 1968, Edgar et al. 2011, Molyneux et al. 2011). Since the liver is a primary source of bioactivation of the pro-toxic dehydroPAs to the toxic didehydroPAs, otherwise referred to as the “pyrrolic” form, then hepatotoxicity is a usual

observation in livestock and humans. In some circumstances, the dehydroPAs or their hepatic metabolites can escape the liver and circulate systemically to cause pneumotoxic sequelae (Pessoa et al. 2013). Some dehydroPAs are also documented genotoxins and can cause cancers in various animal models (Edgar et al. 2011). Humans have been poisoned, with consequent liver-related adverse effects, by dehydroPAs in contaminated grain and in some herbal products. However, it is the potential for low-level, long-term or intermittent dietary exposures that cause, contribute to, or exacerbate chronically developing disease in humans (e.g. various cancers, pulmonary arterial hypertension, and cirrhosis) (Edgar et al. 2015) that has led to international concern about the levels of exposure to these alkaloids in the human diet and dietary supplements.

Over the past 5 to 6 decades, there have been a myriad of international studies of the occurrence and toxicity of dehydroPAs. One of the more commonly studied dehydroPAs, probably because of its facile and abundant isolation from plants such as *Senecio riddellii* (Molyneux et al. 1991), has been the macrocyclic, retronecine-based diester riddelliine (figure 1). As a consequence, it is at present only one of two dehydroPAs, the other being the open chain diester lasiocarpine (figure 1), for which enough data have been accumulated to enable a risk assessment that concluded there were reasonable grounds to classify it as a Group 2B carcinogen, i.e., “possibly carcinogenic to humans” (IARC 2014).

Materials and Methods

Chemicals, Reagents, and Plant Material

Methanol and ethanol were reagent ACS/USP/NF grade (Pharmaco Products, USA). Acetonitrile was HPLC-certified solvent (Honeywell Burdick and Jackson, USA), and water was Milli-Q-purified (18.2 MΩ/cm) (Millipore, USA). The mobile phase additive, formic acid, was “For Analysis” grade (>99%; Acros Organics/Thermo Fisher Scientific, USA). The dehydroPAs heliotrine, riddelliine, retrorsine, seneciphylline, senecionine, and lasiocarpine, and the *N*-oxides of riddelliine, heliotrine, senecionine, and seneciphylline (figure 1), confirmed using NMR spectroscopy and/or HPLC-esi(+)MS, were all sourced from the stocks of extracted and purified or semi-purified pyrrolizidine alkaloids kept by the USDA-ARS Poisonous Plant Research Laboratory. The purity of the alkaloids, determined using HPLC-esi(+)MS, varied from about 90% to >99%. Ammoniated methanol was prepared by bubbling dry ammonia gas through cooled (ice bath) methanol. The resultant saturated ammoniated methanol solution was diluted 1:9 with methanol for use in strong cation exchange, solid phase extraction (SCX SPE). The SCX SPE, HPLC, and guard columns were obtained from Phenomenex (USA). For normal phase chromatography, KP silica gel cartridges (10 g Samplets) and KP-silica gel-HS columns (120 g) were obtained from Biotage (USA).

One dimensional (1D) and 2D (COSY, HSQC, HMBC) ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR data were acquired using a JEOL Eclipse NMR spectrometer using solutions in deuterochloroform (Sigma-Aldrich, USA) and the residual proton in the $CHCl_3$ ($\delta = 7.2$) as the chemical shift reference.

HPLC-MS Analysis

HPLC-esi(+)MS and MS/MS analysis. Using an Agilent 1260 Infinity HPLC System (Agilent Technologies, USA), analytical samples (2 μL) were injected onto a Synergi Hydro RP column (150 x 2 mm, 4μ) fitted with a guard column of similar adsorbent (AC C18, 4 mm diameter x 2 mm) (Security Guard Cartridge system). A gradient flow (400 μL/min) of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) was used to elute sample components from the column. Mobile phase B was held at 3% for 2 min before linearly increasing to 70% by 10 min. After holding at 70% for another 5 min, data acquisition was stopped, and the column was re-equilibrated to 3% mobile phase B over 2 min and held for a further 8 min before the next injection.

Despite the widespread use of riddelliine in toxicology studies, there has been no reference to its potential for degradation or reactivity in solution. A routine high-pressure liquid chromatography-electrospray ionization-positive ion trap mass spectrometry (HPLC-esi(+)MS) analysis of a stored, standard solution of riddelliine in methanol revealed a significant change that was subsequently investigated in more detail.

The eluate from the HPLC column was monitored using a Velos Pro LTQ mass spectrometer (Thermo Scientific, USA) in a 2 scan, positive ion mode, and equipped with a heated electrospray ionization (HESI) source. The first full scan (*m/z* 200-800) was followed by a data-dependent (most intense ion in a peak), collision-

induced dissociation (CID) scan using a generic CID energy of 35%, activation Q of 0.25, and an activation time of 10.0 ms. The capillary temperature was set at 275 °C, the ionization spray voltage at 3.45 kV, the HESI source heater temperature at 305 °C, and the sheath gas flow was 40 units with an auxiliary flow of 5 units.

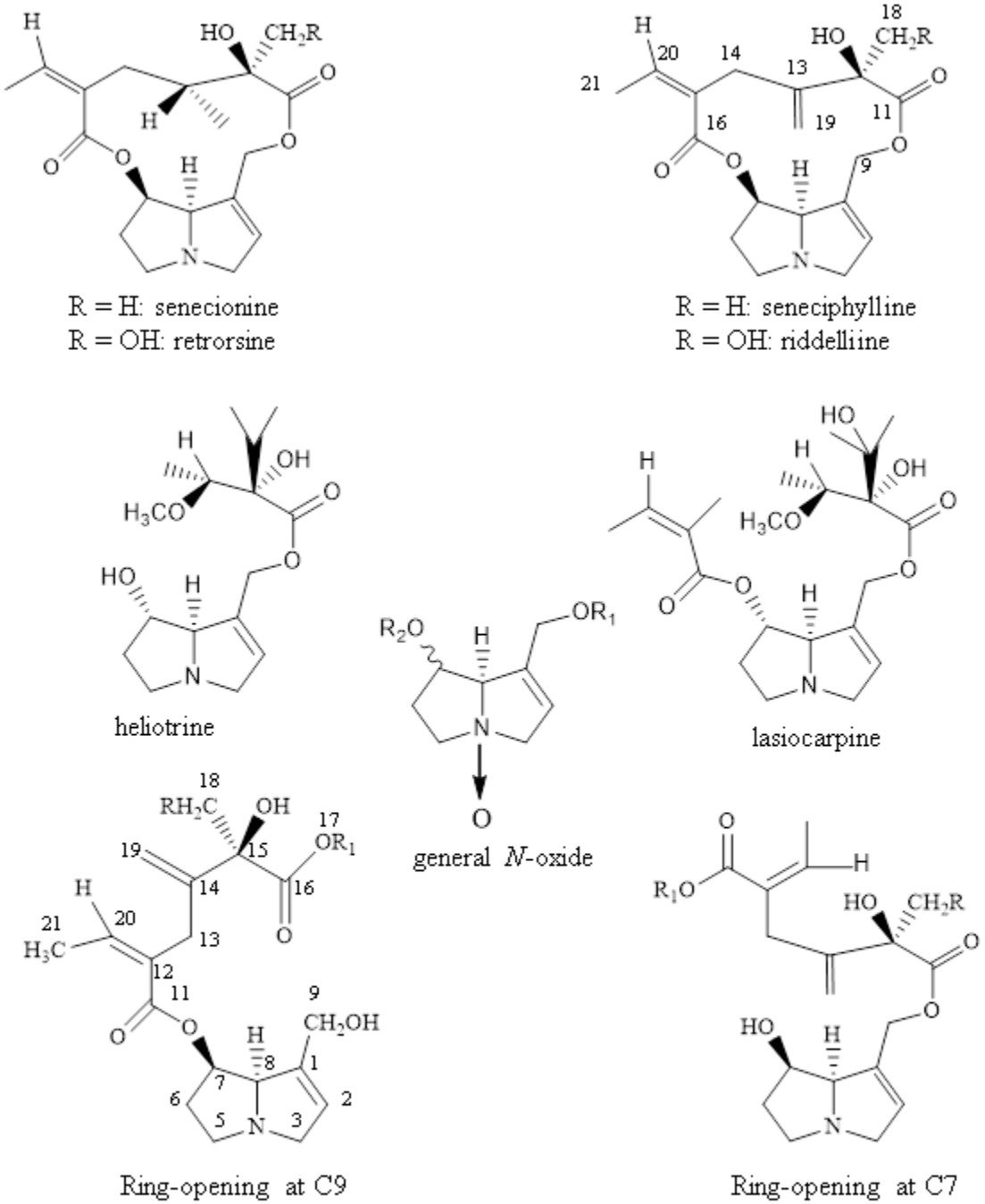

Figure 1. Structures of dehydropyrrolizidine alkaloids as discussed in the text.

HPLC-esi(+)HRMS. High-resolution *m/*z measurements of the dehydroPAs were made using an Ultimate 3000 HPLC (Thermo Scientific, USA) to inject a sample aliquot (2 µL) onto the Synergi Hydro column and elute components using the same acetonitrile/0.1% formic acid gradient as described above. The column effluent was monitored using an Exactive Plus Orbitrap high-resolution mass spectrometer (Thermo Scientific, USA): scan range 100-1,000 Da; resolution 35,000; microscans 1; lock mass 445.12003; sheath gas flow 35; auxiliary gas flow 10; spray voltage 4 kV; capillary temperature 320 °C; S lens RF field 55; auxiliary gas temperature 300 °C; and maximum inject time 250 ms.

Reaction Conditions

Analytical scale. Samples (2-10 mg) of the dehydroPAs and their *N*-oxides were separately dissolved in methanol or ethanol, or in methanol or ethanol containing 0.5% (v/v) of hydrochloric acid (37%) or 0.5% (v/v) or 0.05% (v/v) of ammonium hydroxide (0.88). The solutions were left at room temperature or heated in a sand bath at solvent reflux temperatures. Aliquots (10 µL) were regularly sampled, diluted with lasiocarpine-spiked mobile phase (100 µL), and analysed using HPLC-esi(+)MS and MS/MS.

Preparative scale reaction of riddelliine and isolation of riddelliine-methanol adduct. Riddelliine (ca. 100 mg) was dissolved in methanol (50 mL) containing 0.5% of ammonium hydroxide (0.88). The solution was left at room temperature (ca. 22 °C), and the progress of the reaction was monitored using HPLC-esi(+)MS. After about 2 weeks, or whenever the HPLC-esi(+)MS monitoring indicated near completion of the reaction, the solvent was removed in vacuo by rotary evaporation. The residue was extracted with 0.05 M sulphuric acid (50 mL), filtered, and the consequent aqueous acid solution loaded onto an SCX SPE column (70g/150 mL; 55µm, 70 Å; Strata Giga Tubes) preconditioned by washing with methanol followed by 0.05 M sulphuric acid. The loaded column was washed with water (50 mL) and methanol (100 mL) before the captured alkaloids were eluted from the column using ammoniated methanol. All loadings and column washes were monitored for the dehydroPAs using HPLC-esi(+)MS. The ammoniated methanol fractions containing the alkaloids were pooled and evaporated to dryness in vacuo using a rotary evaporator. The residue was reconstituted in chloroform and, to maintain a narrow band, was added batch wise to a KP-silica gel cartridge (10g Biotage Samplet) and allowed to air dry between additions. The loaded Samplet cartridge was fitted to a KP-silica-HS column (120 g) previously equilibrated with chloroform:10% saturated ammoniated methanol (9:1) using a programmable Isolera 1 flash chromatography system (Biotage, USA). The alkaloid components were eluted from the Samplet-column combination using an isocratic flow (12 mL/min) of chloroform:10% saturated ammoniated methanol (9:1). Collected fractions (5 mL) were analysed using HPLC-esi(+)MS. Fractions with a high (>95%) proportion of the major riddelliine-methanol addition product were pooled and evaporated to dryness (35 mg) for subsequent reconstitution in $CDCl_3$ for NMR analysis.

Results and Discussion

The instability of riddelliine was first observed during a routine HPLC-esi(+)MS analysis of a standard methanol solution of riddelliine (0.9 mg/mL) that had been stored at room temperature (ca. 22°C) for several months. Two additional peaks (one major and one minor) with protonated molecules (MH^+) at *m/z* 382 were observed in the ion chromatogram (figure 2). The mass difference (Δ MH^+ 32 Da) between the riddelliine and the new products implied an addition of methanol to riddelliine. Addition products were also observed when riddelliine was dissolved in ethanol, giving rise to a major and a minor new compound each equivalent to the addition of ethanol (MH^+ *m/*z 396) (figure 3).

Various experiments were conducted to investigate the conditions and salient structural features that might be required for, or that might facilitate, the formation of the addition products. The intent was to accelerate the formation of addition products and allow isolation for NMR spectroscopy analysis. Initially, riddelliine and its *N*-oxide were each dissolved in refluxing methanol or ethanol for several days. Secondly, riddelliine and its *N*-oxide were dissolved in acidified methanol either at room temperature or at methanol-refluxing temperature. Thirdly, riddelliine was dissolved in basified methanol at room temperature. Although formation of the addition products was observed in all cases, it was much faster, going to completion within a week, in the basified methanol or ethanol at room temperature. Therefore, further investigations of

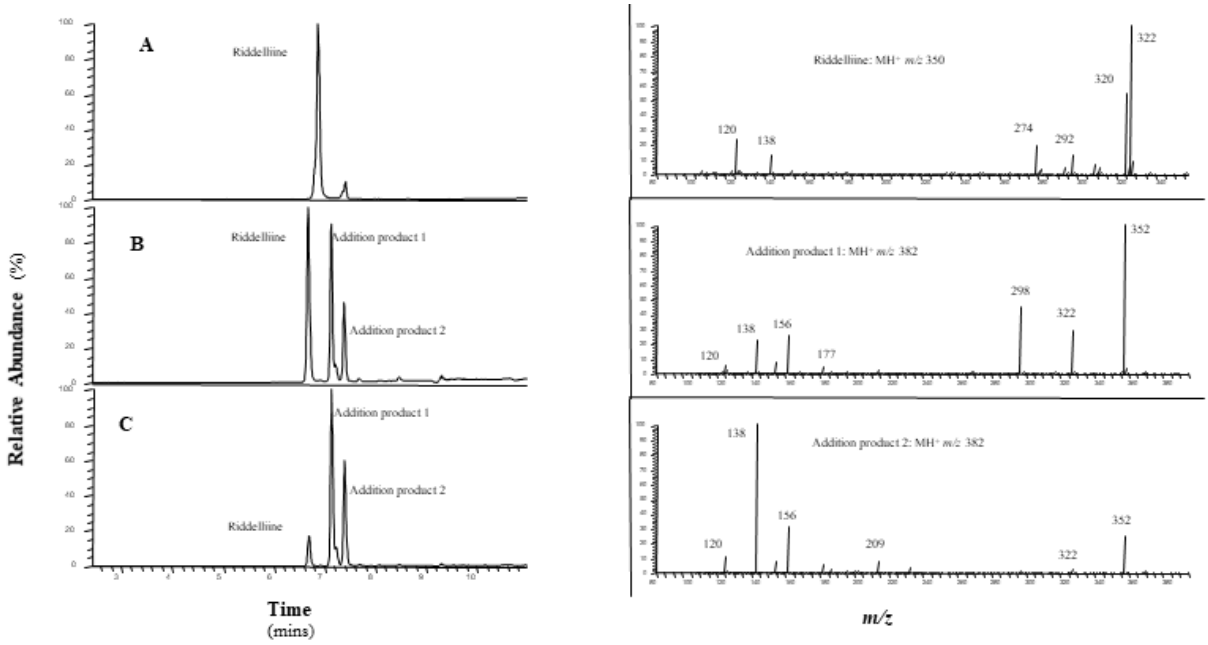

Figure 2. HPLC-esiMS base ion chromatograms (*m/z* 340-400) of A, fresh solution of riddelliine in methanol showing a minor contamination with retrorsine; and B and C, progressive loss of riddelliine and formation of the two addition products. Also shown are the MS/MS fragmentation profiles for riddelliine and the two addition products. *m/z*, mass to charge ratio.

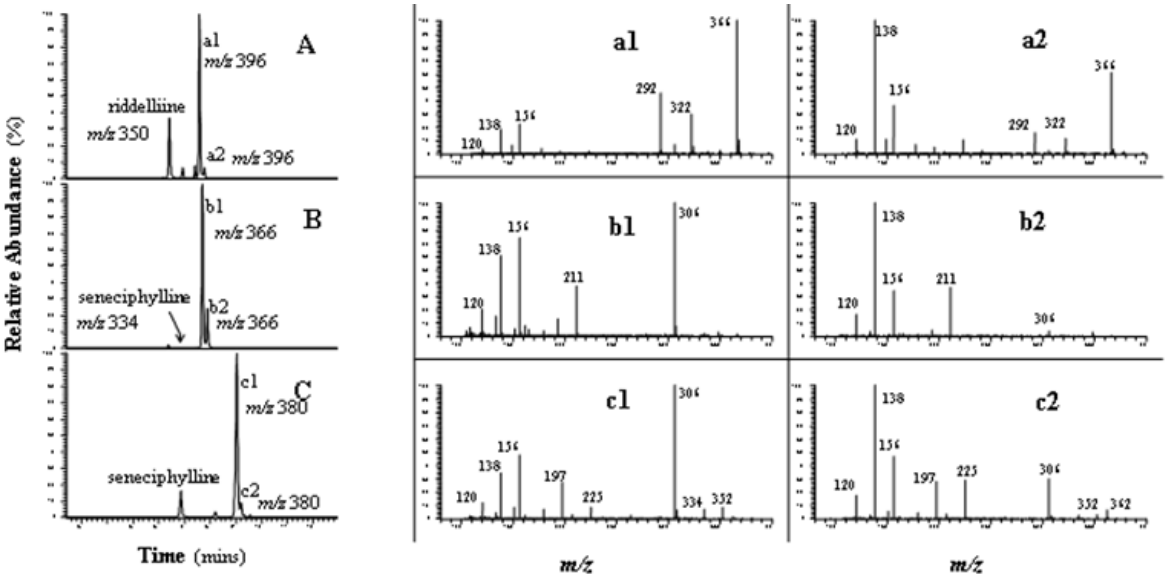

Figure 3. HPLC-esi(+)MS base ion (*m/z* 300-400) chromatograms and MS/MS fragmentation spectra for the ring-opening transesterifications of riddelliine with ethanol (A, a1, a2) and seneciphylline with methanol (B, b1, b2) and ethanol (C, c1, c2). *m/z*, mass to charge ratio.

seneciphylline, analogous to riddelliine with its C13-C19 exocyclic double bond in the macrocyclic diester ring (figure 1), and retrorsine and senecionine, the C13 double bond-reduced analogues of riddelliine and seneciphylline respectively, were conducted using the basic conditions. Also investigated were the reactions of the monoester heliotrine and the *N*-oxides of riddelliine, seneciphylline, senecionine, and heliotrine, in methanol and ethanol, all basified with ammonium hydroxide. Only the macrocyclic diesters with an exocyclic double bond, i.e., riddelliine and seneciphylline, and their *N*-oxides, reacted with the methanol or ethanol (table 1, figures 2 - 4). Neither

the monoester heliotrine nor the macrocyclics without an exocyclic double bond, i.e., retrorsine and senecionine, nor *N*-oxidation per se, i.e., senecionine-*N*-oxide and heliotrine-*N*-oxide (table 1) resulted in any additive or hydrolytic transformation of the parent alkaloids under the experimental conditions examined. Thus, two addition products were observed in each of the reactions of the free base and *N*-oxide forms of riddelliine and seneciphylline with methanol or ethanol. Despite the same molecular formula calculated from the accurately determined molecular masses within any pair of addition products, there were major MS/MS differences in ion formation and relative abundance between the two products indicating a significant difference in their structures (table 1, figures 2-3).

Table 1. HPLC-esi(+)MS, MS/MS, and high-resolution MS data for dehydropyrrolizidine alkaloids and transesterification products discussed in the text. Also shown are the calculated and expected, where applicable, molecular formulae

Dehydropyrrolizidine alkaloid	Expected protonated molecular formula	Retention time (relative abundance) (min)	HRMS (*m/z*)	Calculated protonated molecular formula[a]	Δ (ppm)[a]	MS/MS
riddelliine	$C_{18}H_{24}NO_6$	6.35	350.16172	$C_{18}H_{24}NO_6$	3.9	332(2), 323(8), 322(100), 320(57), 306(5), 304(7), 292(13), 288(4), 276(3), 274(21), 149(2), 138(13), 120(26), 118 (2)
riddelliine-methanol product 1		6.88 (100)	382.18658	$C_{19}H_{28}NO_7$	0	364(1), 352(100), 322(28), 292(47), 209(2), 177(4), 156(25), 149(7), 138(22), 120(5)
riddelliine-methanol product 2		7.13 (15 - 50)	382.18646	$C_{19}H_{28}NO_7$	0.3	364(1), 352(27), 338(0.4), 322(3), 292(3), 227(3), 209(7), 181(2), 177(6), 156(30), 149(8), 138(100), 120(11)
riddelliine-ethanol product 1		7.58 (100)	396.2035	$C_{20}H_{30}NO_7$	3.2	368(10), 367(6), 366(100), 350(2), 338(1), 324(5), 323(2), 322(30), 306(7), 292(46), 223(2), 195(2), 177(4), 156(22), 149(6), 138(18), 120(4)
riddelliine-ethanol product 2		7.78 (8)	396.20324	$C_{20}H_{30}NO_7$	2.4	378(1), 368(2), 367(1), 366(52), 350(1), 324(1), 322(9), 306(2), 292(11), 241(2), 223(10), 195(5), 177(7), 156(35), 149(10), 138(100), 120(11)
riddelliine-water product 1		6.15 (100)	368.16969	$C_{18}H_{26}NO_7$	3.4	350(31), 338(2), 332(2), 324(100), 322(30), 306(95), 156(2), 138(1), 120(0.5)
riddelliine-water product 2		6.54 (15)	368.17026	$C_{18}H_{26}NO_7$	1.8	350(9), 338(0.5), 332(0.5), 324(100), 322(3), 306(31), 156(2), 138(17), 120(2)
seneciphylline	$C_{18}H_{24}NO_5$	7.24	334.16487	$C_{18}H_{24}NO_5$	1.8	334(1), 316(1), 306(100),290(4), 288(22), 274(2), 246(2), 151(4), 138(20), 122(3), 120(32), 118(3)
seneciphylline-methanol product 1		7.7 (100)	366.19168	$C_{19}H_{28}NO_6$	0.05	348(3), 307(8), 306(100), 211(38), 193(13), 179(4), 165(5), 161(8), 157(3), 156(74), 151(5), 139(3), 138(61), 133(15), 120(20), 118(2), 110(3), 108(6)

seneciphylline-methanol product 2		7.9 (25)	366.19165	$C_{19}H_{28}NO_6$	0.04	348(3), 306(4), 211(36), 193(4), 179(1), 165(2), 161(2), 156(34), 151(1), 138(100), 120(60)
seneciphylline-ethanol product 1		9.04 (100)	380.20591	$C_{20}H_{30}NO_6$	3.7	362(1), 352(8), 334(7), 308(6), 306(100), 264(2), 225(8), 207(3), 197(27), 179(7), 161(2), 156(47), 151(8), 138(34), 133(4), 120(12), 118(1), 108(2)
seneciphylline-ethanol product 2		9.2 (10)	380.20613	$C_{20}H_{30}NO_6$	3.1	362(6), 352(2), 334(2), 308(1), 306(21), 225(29), 207(3), 197(26), 179(4), 161(1), 156(43), 151(5), 138(100), 133(2), 120(17)
riddelliine-*N*-oxide	$C_{18}H_{24}NO_7$	7.13	366.15420	$C_{18}H_{24}NO_7$	2.9	348(16), 338(100), 336(69), 318(12), 308(10), 290(15), 274(18), 246(13), 244(6), 154(13), 140(8), 138(15), 137(8), 136(17), 122(6), 121(9), 120(42), 119(31), 118(48)
riddelliine-*N*-oxide-methanol product 1		7.74 (75)	398.18007	$C_{19}H_{28}NO_8$	3.6	368(100), 338(21), 332(11), 308(17), 304(6), 298(4), 288(6), 286(4), 274(5), 272(4), 262(4), 244(5), 209(4), 177(6), 172(25), 154(13), 149(11), 137(8), 136(11), 118(3)
riddelliine-*N*-oxide-methanol product 2		8.08 (100)	398.18014	$C_{19}H_{28}NO_8$	3.4	380(41), 368(100), 352(3), 350(3), 348(1), 339(1), 338(55), 308(1), 172(8), 156(2), 155(2), 154(4), 138(15), 137(3), 136(9), 120(4)
riddelliine-*N*-oxide-ethanol product 1		8.23 (50)	412.19565	$C_{20}H_{30}NO_8$	3.6	384(10), 383(5), 382(100), 346(9), 338(24), 322(6), 308(24),304(7), 302(5), 286(4), 274(6), 244(5), 223(4), 195(4), 177(7), 172(29), 154(13), 149(12), 137(5), 136(11)
riddelliine-*N*-oxide-ethanol product 2		8.50 (100)	412.19565	$C_{20}H_{30}NO_8$	3.6	394(42), 383(5), 382(100), 376(2),366(5), 364(3), 340(2), 339(3),338(61), 322(3), 308(1), 172(8), 156(1), 155(1), 154(4), 138(11), 137(2), 136(10),120(4)
seneciphylline-*N*-oxide	$C_{18}H_{24}NO_6$	8.71	350.15870	$C_{18}H_{24}NO_6$	4.7	332(16), 322(100), 288(12), 274(7), 246(27), 244(11),218(8),178(7), 167(10), 154(17), 151(7), 138(13), 136(16), 122(13), 121(13), 120(44), 119(28), 118(55), 117(8), 106(8)
seneciphylline-*N*-oxide –methanol product 1		8.8 (25)	382.18507	$C_{19}H_{28}NO_7$	3.9	322(73), 302(11), 288(77), 270(28), 262(12), 260(13), 244(46), 226(11), 216(15), 211(63),193(19),172(100), 161(14), 154(49), 151(11), 137(27), 136(41), 133(24), 120(11), 118(13)
seneciphylline-*N*-oxide –methanol product 2		9.09 (100)	382.18483	$C_{19}H_{28}NO_7$	4.6	364(95), 322(100), 288(1), 262(1), 252(1), 211(1),

						172(10), 156(1), 155(3), 154(7), 138(17), 137(4), 136(12),133(1), 120(4), 118(1)
seneciphylline-*N*-oxide –ethanol product 1		9.31 (42)	396.20125	$C_{20}H_{30}NO_7$	2.5	368(14), 350(10), 322(100), 316(10), 297(10), 288(80), 270(31), 260(12), 244(36), 226(10), 225(33), 216(11), 197(52), 179(19), 172(96), 154(42), 151(24), 137(22), 136(34), 133(11), 118(11)
seneciphylline-*N*-oxide –ethanol product 2		9.55 (100)	396.20119	$C_{20}H_{30}NO_7$	2.6	378(100), 350(5), 322(100), 288(1), 262(1), 252(1), 197(1), 172(8), 156(1), 155(2), 154(5), 151(1), 138(15), 137(3), 136(10), 120(3), 118(1)
retrorsine	$C_{18}H_{26}NO_6$	6.92	352.17560	$C_{18}H_{26}NO_6$	1.2	352(2), 324(100), 322(28), 308(3), 306(7), 304(3), 294(2), 290(3), 276(17), 220(3), 169(2), 151(4), 138(17), 122(3), 121(2), 120(25), 118(2),103(2)
senecionine	$C_{18}H_{26}NO_5$	7.77	336.18094	$C_{18}H_{26}NO_5$	0.5	336(2), 308(100), 292(4), 290(15), 274(3), 238(2), 220(3), 153(5), 140(1), 138(27), 120(37), 118(4), 103(2)
senecionine-*N*-oxide	$C_{18}H_{26}NO_6$	9.47	352.17563	$C_{18}H_{26}NO_6$	1.1	334(14), 324(85),254(25), 248(25),246(57), 220(100), 218(22), 202(9), 154(16), 152(10), 138(13), 137(9), 136(25), 122(10), 121(27), 120(44), 119(28), 118(69), 117(9), 106(11)
heliotrine	$C_{16}H_{28}NO_5$	7.76	314.19577	$C_{16}H_{28}NO_5$	3.1	296(2), 282(1), 156(4), 152(1), 138(100), 120(3), 96(4), 94(2)
heliotrine-*N*-oxide	$C_{16}H_{28}NO_6$	8.05	330.19122	$C_{16}H_{28}NO_6$	1.3	298(4), 172(100), 155(2), 154(4), 138(7), 137(3), 136(5), 124(1), 94(1)
lasiocarpine	$C_{21}H_{34}NO_7$	9.49	412.233579	$C_{21}H_{34}NO_7$	0.1	394(25), 336(100), 322(2), 312(1), 254(1), 238(5), 220(32), 120(8)

[a]Calculated using ChemCalc (http://www.chemcalc.org/mf_finder/mfFinder_em_new).
MS, mass spectrometry; MS/MS, tandem mass spectrometry; HRMS, high resolution mass spectrometry

It has been previously noted that the MS/MS fragmentation spectra of macrocyclic dehydroPAs with a hydroxymethyl substituent at C12, i.e., α to the C9 ester carbonyl (e.g. riddelliine structure, figure 1) can show a significant loss of 30 Da in addition to the more usual loss of 28 Da from the protonated macrocyclic dehydroPA diester molecule (Boppré et al. 2008, Colegate and Gardner 2008). However, although retrorsine, riddelliine, and riddelliine-*N*-oxide showed this characteristic pair of fragment ions, the riddelliine and riddelliine-*N*-oxide addition products with methanol and ethanol did not, with the exception of the minor product of riddelliine-*N*-oxide with ethanol that showed a 10% relative abundance of MH^+ -28 at *m/z* 384 (table 1). Instead, they simply showed a loss of 30 Da from the MH^+ i.e., *m/z* 382 → *m/z* 352 and *m/z* 396 → *m/z* 366, for the methanol and ethanol addition products to riddelliine respectively, or *m/z* 398 → *m/z* 368 and *m/z* 412 → *m/z* 382, for the methanol and ethanol addition products to riddelliine-*N*-oxide, respectively. This observation implies a significant alteration of the structure involving the C9 and/or C7 ester linkages thereby facilitating alternative fragmentations. This change in MS/MS fragmentation was not observed with seneciphylline or its *N*-oxide and their addition products with methanol or ethanol since seneciphylline does not

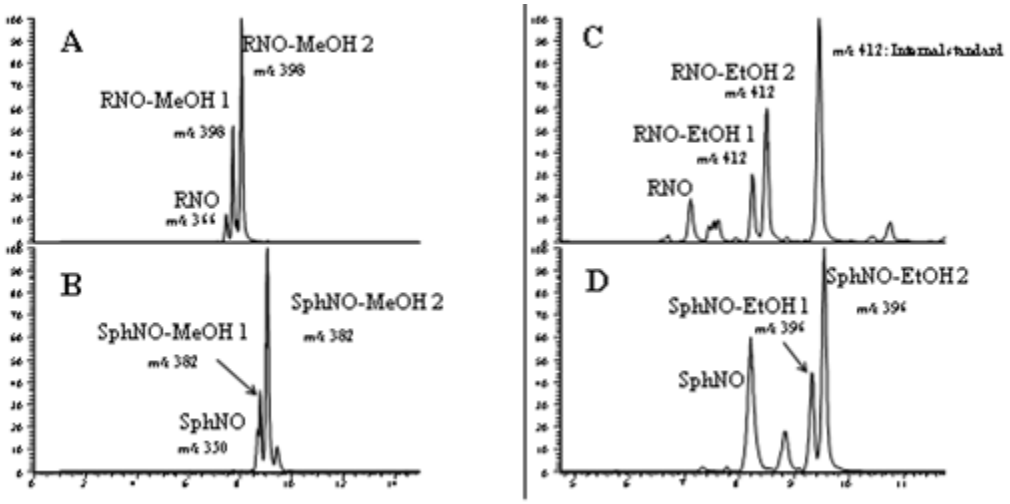

Figure 4. HPLC-esi(+)MS base ion chromatograms of the reactions of riddelliine-*N*-oxide (RNO) with methanol (MeOH) (A) and ethanol (EtOH) (B); and of seneciphylline-*N*-oxide (SphNO) with methanol (MeOH) (C) and ethanol (EtOH) (D). Each of the alkaloids shows two addition products each with the same MH^+ (labeled on peaks as *m/z*) but with different MS/MS fragmentation spectra (table 1). *m/z*, mass to charge ratio.

possess the requisite hydroxymethyl substituent (figure 1, table 1).

While all the initial experiments were conducted on a small scale that allowed HPLC-esi(+)MS and MS/MS monitoring, the reaction of riddelliine with methanol was also conducted on a larger scale, and the major riddelliine-methanol addition product was separated and purified in a two-stage process. The first stage involved SCX SPE to concentrate the alkaloidal material from the reaction mixture. The dried alkaloidal fraction, reconstituted in chloroform, was then chromatographed on silica gel to separate the addition product from unreacted riddelliine, the minor addition product, and minor contaminants. Unambiguous structure elucidation of the major riddelliine-methanol addition product was achieved using 1D (^{1}H, ^{13}C) and 2D (COSY, HSQC, HMBC) NMR spectroscopy (tables 2 and 3, figures 5-7).

All direct carbon-hydrogen bonds were readily established using the heteronuclear single quantum coherence (HSQC) NMR experiment (figure 5, table 2), while ^{1}H-^{1}H couplings were established via the 2D correlation (COSY) NMR spectrum (figure 6, table 3). It was evident that the exocyclic methylene group of the riddelliine (C13→C19, riddelliine in figure 1) had been retained in the methanol addition product (C14→C19, riddelliine-methanol addition product in figure 1). It was also determined that a methoxy group had been added (^{1}H17, δ 3.79; ^{13}C17, δ 53.33) for which the heteronuclear multiple bond correlation (HMBC) NMR experiment (figure 7, table 3) showed its protons (numbering shown on riddelliine-methanol addition product in figure 1) correlated only to the downfield carbonyl (C16, δ 173.92). Also correlated to this downfield carbonyl are the C18 protons and, weakly, H19d. There are no correlations between the H9 protons and either carbonyl carbon (C11 or C16). The upfield carbonyl (C11, δ 166.94) is correlated to H21 (vinyl methyl), H20 (alkene proton), H13d, and 13u (methylene carbon protons) and, very weakly, to H7, clearly establishing its sub-structure environment. These observations confirm a ring-opening transesterification between C9 and C11 of the riddelliine molecule to form the C7 monoester of retronecine with methylriddelliic acid (figure 1).

Although not unambiguously determined using NMR spectroscopy, based on the MS, MS/MS, and HRMS data, it is reasonable to expect that ethanol causes a ring-opening transesterification of riddelliine to yield the ethyl ester. Similarly, it is reasonable to deduce from the data that seneciphylline reacts in the same way as riddelliine, resulting in the major production of the C7 monoester of retronecine with methyl(or ethyl)senecinic acid (figure 1). Furthermore, HPLC-esi(+)MS analysis ascertained that riddelliine is stable in water containing 0.5% (v/v) of HCl (37%) but hydrolyses rapidly, starting within a couple of hours and going to completion over a week at room temperature, in 0.5% (v/v) of ammonium hydroxide (0.88). Two products were formed, the major one eluting just before, and the minor product just after,

the riddelliine peak. Both displayed protonated molecules at *m/z* 368 and accurate masses corresponding to ring-opening hydrolysis of a single ester bond in riddelliine. Similar to the formation of the methanol and ethanol ring-opening addition products, the MS/MS fragmentation spectra (table 1) indicated that the major product was the C7 monoester of retronecine with riddelliic acid while the minor product was the analogous C9 monoester.

Table 2. Carbon-13 (^{13}C) and proton (^{1}H) nuclear magnetic resonance spectroscopy chemical shifts for the major methanol transesterification product resulting from ring-opening at C9 ester of riddelliine (figure1)

Carbon[a]	Chemical shift (ppm)	Proton	Chemical shift (ppm)
1	139.18	-	
2	123.41	2	5.64, bs
3	62.91	3d	3.92, m
		3u	3.38, m
5	53.64	5d	3.34, m
		5u	2.67, m
6	34.79	6d and u	2.05, m
7	74.46	7	5.38, bs
8	75.84	8	4.32, bs
9	59.94	9d and u	4.11, bs
11	166.94	-	-
12	129.63	-	-
13	36.19	13d and u	3.02, ABq, J=10.9, 16.2
14	145.14	-	-
15	80.25	-	-
16	173.92	-	-
17	53.33	17	3.79, 3H, s
18	66.46	18d	3.98, d, J=11.34
		18u	3.73, d, J=11.37
19	114.4	19d	5.23, s
		19u	4.94, s
20	140.5	20	6.04, q, J=7.45
21	15.95	21	1.96, d, J=7.4

[a]Numbering according to figure 1. All assignments confirmed using gradient-enhanced HSQC NMR spectroscopy: s, singlet; bs, broad singlet; d, doublet; q, quartet; ABq, AB quartet; m, multiplet.

Table 3. Proton-proton correlations (COSY) and proton-carbon multiple bond correlations (HMBC) for the major methanol addition product (MH^+ *m/z* 382) resulting from ring-opening transesterification at the C9 ester of riddelliine (figure1)[a]

Proton	Correlated protons	Correlated carbons
2	3u(w), 3d(m), 9d and u(m), 8(w)	1(w), 3(m), 8(m), 9(w)
3d	3u(s), 2(w), 9(m)	1(w), 2(wm)
3u	3d(s), 2(w), 9(m), 8(s)	1(w), 2(wm)
5d	5u(s), 6d and u(s)	6(w), 3(w), 7(wm), 8(wm)
5u	5d(s), 6d and u(s)	6(ms), 3(ms)
6	5d(m), 5u(s), 7(m)	8(w), 7(w)
7	6d and u(m), 8(s)	5(wm), 2(w), 11(vw)
8	3u(s), 7(m), 2(w)	1(w)
9d and u	2(m), 3u(s), 3d(m)	1(s), 2(s), 8(m)
13d and u	13u and 13d(s), 21(m), 19u(m), 19d(w)	11(ms), 12(s), 14(s), 15(m), 19(s), 20(s)
18d	18u(s)	14(m), 15(w), 16(m), 18(w)
18u	18d(s)	14(m), 15(s), 16(s)
19d	13d and u(m)	13(s), 14(m), 15(s), 16(w)
19u	13d and u(m)	13(s), 14(wm), 15(s), 16(vw)
20	21(s), 13(w)	11(ms), 21(m), 13(ms)
21	13d and 13 u(m), 20(s)	11(m), 12(s), 13(w), 14(m), 20(s)
OMe	-	16(s)

[a]Proton and carbon numbers refer to figure 1.

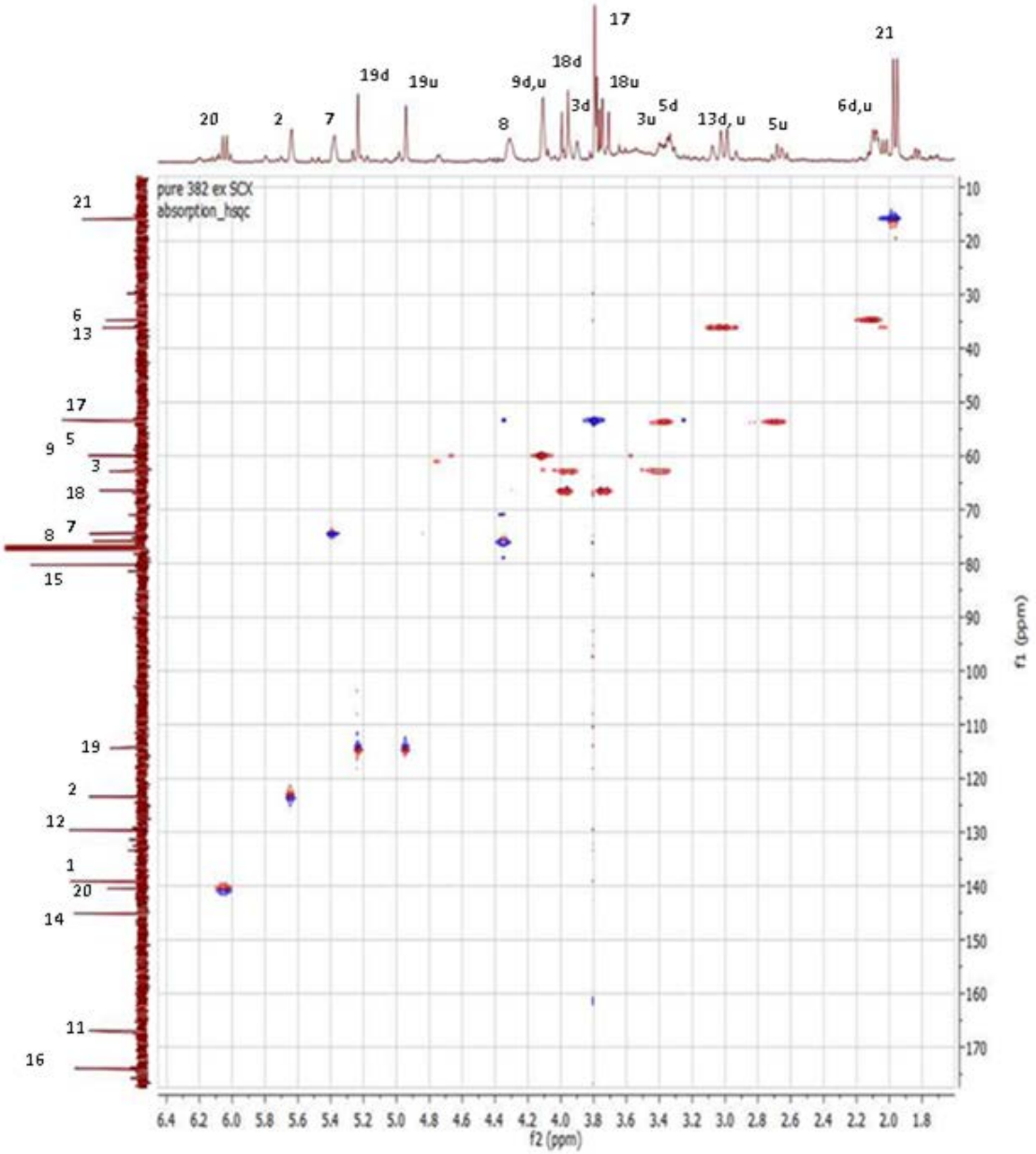

Figure 5. A gradient-enhanced ^{1}H- ^{13}C heteronuclear single quantum coherence (HSQC) NMR spectrum of the major riddelliine-methanol addition product resulting from a ring-opening transesterification at the C9 ester of riddelliine (figure 1).

Each of the riddelliine and riddelliine-*N*-oxide addition products with methanol or ethanol exhibits a facile MS fragmentation of 30 Da to yield a different base ion, i.e., *m/z* 352 and 356 for the methanol and ethanol adducts, respectively, with riddelliine and *m/z* 368 and 382 for the methanol and ethanol adducts, respectively, with riddelliine-*N*-oxide. This most likely represents a McLafferty-type elimination of formaldehyde from the hydroxymethyl-carbonyl entity (figure 9). Without this α-hydroxymethyl-carbonyl entity, each pair of seneciphylline and seneciphylline-*N*-oxide addition products fragments differently, i.e., MH^+ - 60 for methanol adducts or MH^+ - 74 for ethanol adducts, to produce the same ion, *m/z* 306 and *m/z* 322 for seneciphylline and its *N*-oxide, respectively (table 1, figure 8). This most likely represents hydrogen transfer to the terminal carbonyl oxygen from the adjacent hydroxyl group thereby releasing the entire terminal ester function (figure 9).

It is noted that with riddelliine and seneciphylline, the major product with methanol or ethanol eluted before the minor product, whereas the reverse was observed with the *N*-oxides (table 1, figures 2-4). In every case, the minor product of the reaction of methanol or ethanol with riddelliine and

seneciphylline shows a base ion peak at *m/z* 138 that is consistent with a C9 monoester (Pedersen and Larsen 1970, Colegate et al. 2012) and as reported herein for heliotrine (figure 1, table 1). The increased abundance of fragments with higher *m/z* observed for the major addition products, indicating an increased tendency for intra-necic acid fragmentation, supports them all being C7 monoesters similar to the structure unambiguously described for the major riddelliine-methanol addition product (figure 1). In contrast to the observations of the riddelliine and seneciphylline adducts, a similar examination and interpretation of the MS/MS fragmentation of the addition products with the *N*-oxides (figure 8, table1) indicates that the minor products are C7 monoesters, eluting before the major C9 monoester products in each case. This interpretation is supported by the increased fragmentation, including the much higher relative abundance of *m/z* 172, in the MS/MS fragmentation spectra of the minor products (figure 8 A, C, E, and G), and by the presence of *m/z* 138 only in the MS/MS fragmentation spectra of the major products (figure 8 B, D, F, and H). Thus, it seems that in both cases of methanol or ethanol addition to the free base or *N*-oxides of riddelliine and seneciphylline, the C7 monoester product elutes before the C9 isomer. However, the relative abundances of the two isomers formed from the free bases are reversed when formed from the *N*-oxides. There was no evidence for complete hydrolysis of the alkaloids to the retronecine base.

These transesterification addition products are previously undescribed dehydroPAs with unknown toxicities that may contribute to or mitigate against toxic effects during a toxicological evaluation of riddelliine, seneciphylline, and, perhaps, similar macrocyclic dehydroPAs that are susceptible to ring-opening transesterification. It also poses questions about the potential for such ring-opening in vivo and how it may contribute to or mitigate against toxicity by affecting absorption, distribution, metabolism, and excretion of the alkaloids.

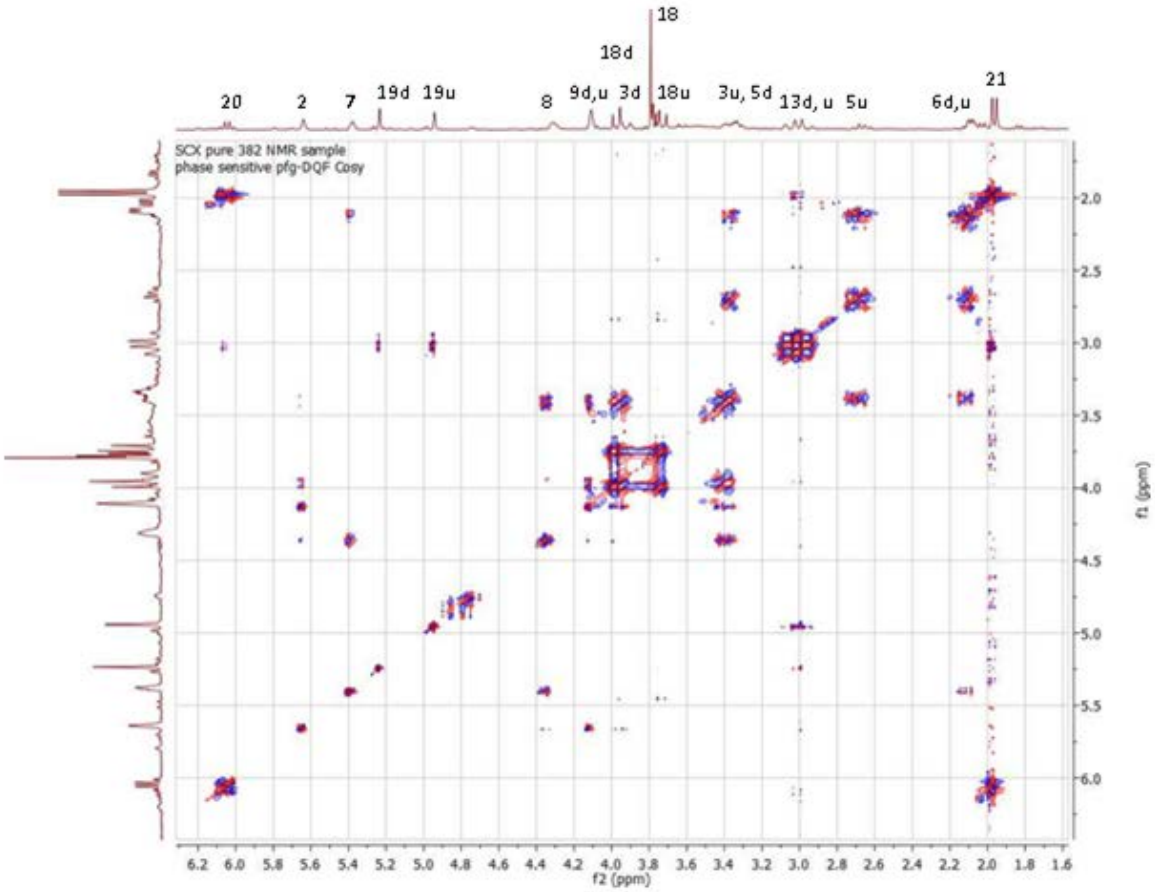

Figure 6. Phase-sensitive gradient enhanced $^{1}H - {}^{1}H$ correlation NMR (COSY) of the major riddelliine – methanol addition product resulting from a ring-opening transesterification at the C9 ester of riddelliine (figure1).

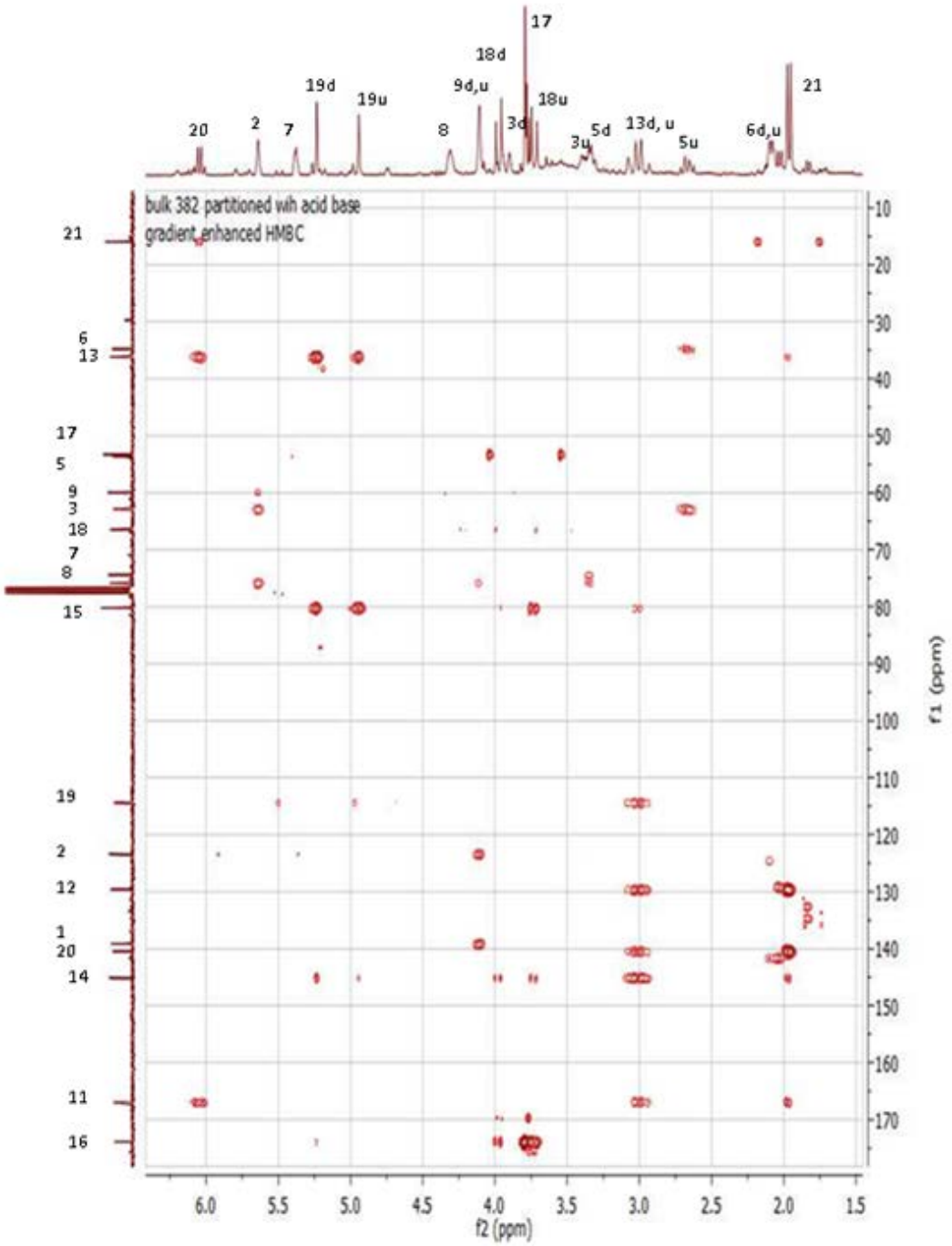

Figure 7. A gradient-enhanced ^{1}H- ^{13}C heteronuclear multiple bond correlation (HMBC) NMR spectrum of the major riddelliine-methanol addition product resulting from a ring-opening transesterification at the C9 ester of riddelliine (figure 1).

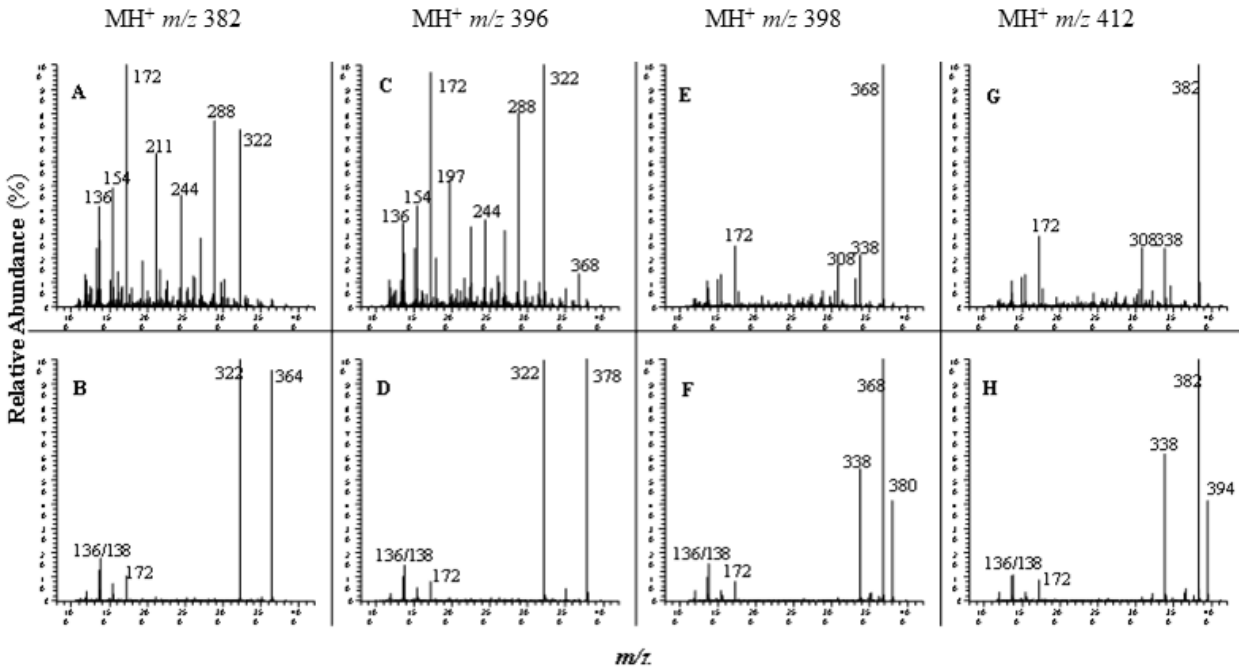

Figure 8. MS/MS fragmentation spectra for the ethanol (EtOH) and methanol (MeOH) addition products of riddelliine-*N*-oxide (RNO) and seneciphylline-*N*-oxide (SphNO) (table 1). A, SphNO-MeOH (1); B, SphNO-MeOH (2); C, SphNO-EtOH (1); D, SphNO-EtOH (2); E, RNO-MeOH (1); F, RNO-MeOH (2); G, RNO-EtOH (1); H, RNO-EtOH (2). *m/z*, mass to charge ratio.

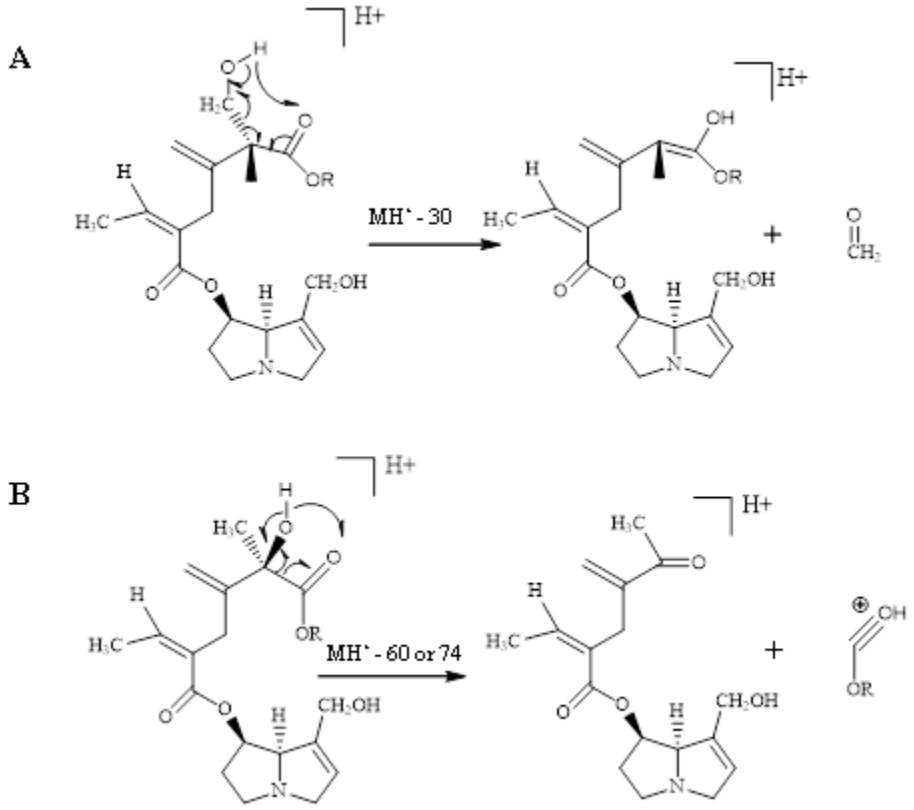

Figure 9. Proposed initial MS/MS fragmentation for A, methanol and ethanol addition products with riddelliine and its *N*-oxide and B, methanol and ethanol addition products with seneciphylline and its *N*-oxide.

Acknowledgments

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Fiddleneck (*Amsinckia intermedia* Lehmann Boraginaceae): Toxicity in Cattle Potentiated by Burrow Weed (*Isocoma acradenia*)

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Abstract

All *Amsinckia* species contain pro-toxic dehydropyrrolizidine alkaloids; however, toxicoses of livestock have only been reported from four (i.e., *A. lycopsoides*, Lehm; *A. menziesii*, Lehm; *A. intermedia*, Fisch. & C.A. Mey; and *A. tessellata*, Gray). A recent case of poisoning and death of 15 out of approximately 150 pregnant cows on an Arizona rangeland is reported. Field investigation of the poisoning site implicated fiddleneck (*Amsinckia intermedia*) and burrow weed (*Isocoma acradenia*) as both were present with evidence of being grazed. Necropsy and subsequent histopathology of two dead cows supported the diagnosis of fiddleneck poisoning, likely potentiated by burrow weed ingestion, as the cows had hepatic necrosis with hemorrhage and minimal biliary hyperplasia indicative of pyrrolizidine alkaloid (fiddleneck) poisoning combined with both mycocardial and skeletal muscle necrosis indicative of burrow weed poisoning. Analysis of whole blood of two additional cows for pyrrolizidine alkaloid pyrrole adducts was positive. Chemical characterization of the dehydropyrrolizidine alkaloids identified in the fiddleneck samples collected included the monoester retronecines, lycopsamine and intermedine along with their 7- and 13- acetyl derivatives, and their respective *N*-oxides which collectively comprised 2% of the plant dry weight. While tremetone and other benzofuran ketones totaled 1.25 µg/mg dry plant in the burrow weed.

Key words: *Amsinckia*, burrow weed, *Isocoma*, fiddleneck, hepatotoxicity, myotoxicity

Introduction

Commonly referred to as “tarweeds” or “fiddlenecks” because of their distinctive scorpioid cymes (figure 1), the *Amsinckia* spp. are adapted to a variety of soil types and are common invasive weeds on many western U.S. rangelands (Kingsbury 1964, Burrows and Tyrl 2013). *Amsinckia*-related poisoning involves hepatotoxic effects, including fibrosis of the liver, and associated secondary effects such as jaundice, ascites, and photosensitization. *Amsinckia*-related poisoning has been reported to occur when animals are fed grain screenings contaminated with *Amsinckia* seed (McCulloch 1940a). Clinically, animals have severe jaundice and ascites and may develop photosensitization. Reported microscopic lesions include hepatic necrosis with biliary hyperplasia and fibrosis. If animals survive these changes, they can develop cirrhosis and ultimately liver failure.

Poisoning of pigs, horses, and cattle have been reported, with horses being extremely sensitive, while fowl, sheep, and mules are reported to be quite resistant

Figure 1. *Amsinckia intermedia* (original photo by K.E. Panter) and line drawing of *Amsinckia* showing the scorpioid cymes (original line drawing by Holly Broom-Hyer).

to poisoning from pyrrolizidine alkaloids in general and *Amsinckia* seeds specifically (Cheeke and Shull 1985, Burrows and Tyrl 2013). The disease was first described in horses as "walking disease," characterized by aimless wandering, and reported in the Pacific Northwest in 1925 (Kalkus et al. 1925), although farmers and ranchers had recognized the disease some 30 years or more before that (Kingsbury 1964). Other descriptive terms for the hepatic disease include hard liver disease, Walla Walla hard liver disease, protein poisoning, and winter wheat poisoning (Burrows and Tyrl 2013). The poisoning disease is linked to pyrrolizidine alkaloid-induced hepatotoxicity.

Broadly speaking, the pyrrolizidine alkaloids (PAs) are a diverse class of secondary metabolites comprising two fused 5-membered rings with a nitrogen at a bridgehead. The pro-toxic 1,2-dehydropyrrolizidine alkaloid esters (DHPAs) are a specific subclass that, following in vivo metabolic activation (especially by hepatocytes), are potent alkylators of biomacromolecules, thereby leading to the toxic sequelae. Colegate et al. (2013) reported on the phytochemical analysis of the *Amsinckia intermedia* collected in Arizona and Washington and screened plants beginning at the time of poisoning in this case and monthly thereafter. They determined that the alkaloid levels changed over time, and as the plants matured, the ratio of lycopsamine to its *N*-oxide varied from a low of 0.01 in February to a high of 1.06 in early May.

Until the mid-1900s, when herbicide technology emerged and application practices controlled broad leaf weeds, poisoning in livestock from contaminated grains was common (Cheeke and Shull 1985). With the advent of modernized cultivation and harvesting techniques, as well as current research advances, poisoning in pigs is now rare, and poisoning in horses and cattle is significantly reduced. Nonetheless, poisoning cases continue to occasionally occur and are usually associated with contaminated hay or lack of good-quality forage on the rangeland as presented in this case report.

Burrow weed, jimmy weed, or rayless goldenrod (*Isocoma* spp.) intermittently poison livestock in many areas of the southwestern United States. Poisoning is characterized by muscle weakness that is most often seen as reluctance to move and trembling with forced exertion. Some poisoned animals may develop tachypnea and tachycardia with ascites and hydrothorax. Poisoning generally occurs in late fall or winter when other forages are limited. The burrow weed toxin or one of its metabolites is excreted in the milk causing secondary poisoning of nursing neonates often without apparent maternal toxicity (Davis et al. 2013a,b). Poisoning has been reproduced experimentally, and historically the toxins have been identified as benzofuran ketones, tremetone, hydroxytremetone, and 3-oxyangeloyl-tremetone, and several additional minor compounds (Lee et al. 2009, 2010). However, poisoning is sporadic and often does not seem to be related to the dose of the proposed toxins where tremetone has been present in toxic plant populations of *Isocoma* spp. and *Ageratina altissima* (white snakeroot) (Stegelmeier et al. 2010; Davis et al. 2013a,b, 2016).

In this case report, we describe (1) a recent case of *Amsinckia intermedia*-associated poisoning that may be potentiated by *Isocoma acradenia* of cattle in Arizona, (2) a phytochemical analysis of the *Amsinckia* and *Isocoma* species collected at the site and time of the poisoning outbreak, and (3) a chemical comparison of a second *Amsinckia intermedia* collection in Washington State where the plant is utilized by cattle but not associated with poisoning.

Materials and Methods

Case Overview

A case of 15 dead cows from a herd of approximately 150 was reported to the USDA-ARS Poisonous Plant Research Laboratory (PPRL), Logan, UT, in February 2012. The local veterinarian necropsied two cows, and blood samples were taken from two additional cows.

Cattle were moved to other pastures when the dead cows were discovered, and further losses were not reported. Tissue samples and blood serum samples were submitted to PPRL for histopathological and chemical analysis. Personnel from PPRL went to the range site approximately 25 miles north of Kingman, AZ, to evaluate the pastures. The rangeland where the cows died was surveyed for plant species that could be responsible for the death losses. The range was in poor condition with multiple forb species emerging but very little grass available for grazing. Immature fiddleneck and burrow weed with green leaves had been extensively grazed. Forbs were identified, recorded, and plant specimens collected for voucher filing and chemical analysis. All voucher specimens were filed at the Poisonous Plant Research Laboratory Herbarium for future reference.

Phytochemical Analysis

Amsinckia plant samples collected for chemical analysis were air dried, ground to pass a 5 mm screen, extracted with methanol, and analyzed according to Colegate et al. (2013). The *Isocoma acradenia* samples were also dried, ground, and analyzed as previously described (Lee et al. 2009).

Taxonomic Summary Revision

Plant voucher specimens were sent to Stanley Welsh at the Brigham Young University (BYU) Herbarium for identification. Welsh reported that there is much confusion and misinformation about the classification of the *Amsinckia* species in general. He suggested that a summary revision of the *Amsinckia* species would be a good companion paper to this case report. Therefore, a revision of the taxonomic classification of *Amsinckia* spp. is published as a companion paper to this case report.

Results and Discussion

Case Overview

Fifteen pregnant cows out of a herd of approximately 150 died while grazing on pastures north of Kingman, AZ, in February 2012. The local veterinarian necropsied two cows (case #12-029 and case #12-049) and found yellow discoloration of many serosal and mucosal epithelia and fatty tissues (severe icterus). The liver was firm and yellow, and on cut sections, it had an accentuated lobular pattern. The gall bladder was enlarged and bile filled. These lesions were consistent with DHPA-induced liver disease and provided the impetus for further investigation. Liver and blood serum samples were sent to the USDA-ARS Poisonous Plant Research Laboratory (PPRL) for further analysis. Field investigation revealed poor range conditions and an abundance of fiddleneck (*Amsinckia intermedia*) plants 3 to 6 inches tall. Earlier rains had caused a flush of fiddleneck growth, and there was extensive evidence of grazing of the new lush plants (figure 2). Admixed within the fiddleneck were numerous clusters of burrow weed with young green leaves that also had been grazed.

Figure 2. Grazed *Amsinckia* plants shortly after the cattle had been removed from the range.

Other plants present included cheese weed (*Hymenoclea salsola*), fourwing saltbush (*Atriplex canescens*), Big Galleta (*Hilaria regida*), Arizona popcornflower (*Plagiobothrys arizonicus*), and a few unidentifiable forbs. There was very little grass growing, and what was there had been heavily grazed. It was determined that poor range conditions and green growing fiddleneck was probably the cause of the poisoning. Fiddleneck and burrow weed samples were taken for voucher specimens at the time of the investigation and subsequently by the extension agent. Fiddleneck voucher specimens were collected, pressed, and filed in the Poisonous Plant Research Laboratory Herbarium (voucher #4378 collected February 16, 2012; voucher #4360 collected March 28, 2012; and voucher #4394 collected April 12, 2012). Burrow weed voucher specimens were also collected, identified as *Isocoma acradenia*, and filed with the Poisonous Plant Research Laboratory Herbarium (voucher #s 4426-4428 collected on February 16, 2012). Vouchers were sent to BYU Herbarium in Provo, UT, and Dr. Stanley Welsh confirmed that all were *Amsinckia intermedia* or *Isocoma acradenia*.

Histopathology of the liver samples from case 12-029 revealed severe hepatocellular degeneration and necrosis. Most hepatocytes were swollen with extensive accumulations of intracellular lipid disruption

of hepatic cords. The degenerative hepatocytes were enlarged, and small numbers were fused and formed syncytia. The sinusoids and central veins were dilated and filled with blood. The myocardium had multiple small necrotic foci characterized by myocyte swelling, coagulation, and clumping of myofibers with focal monocytic and lymphocytic inflammation (figure 3). Skeletal muscles also had rare myofiber swelling and hypereosinophilia (figure 4). The second cow (case 12-049) was severely autolytic, which obscured many changes. The skeletal muscle had myofiber necrosis characterized by swelling and hypereosinophilia with myofiber clumping. There was minimal inflammation and increased numbers of nuclei suggestive of regeneration. Similar small foci of degeneration were present in the myocardium. The liver was congested, and the sinusoids were dilated and filled with blood (figure 5). These histologic changes are suggestive of both DHPA and *Isocoma* spp. intoxication.

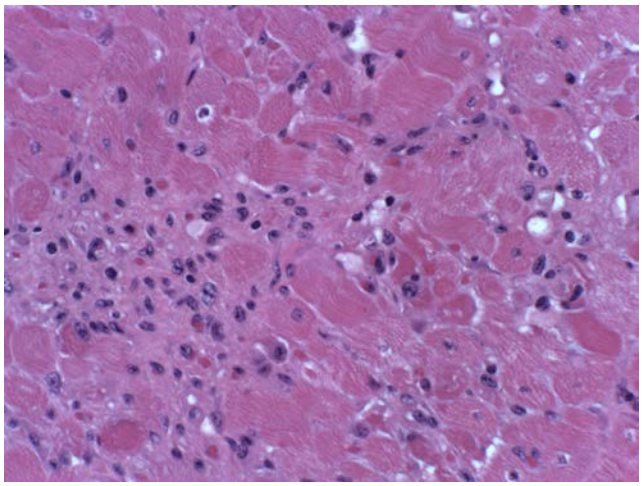


Figure 3. Myocardium from cow 12-029. Notice the focally extensive myocyte degeneration and necrosis seen as myofiber loss of striation, swelling, hypereosinophilia, and coagulation of proteins. The interstitium is expanded with fibrous connective tissue and monocytic inflammation.

Liver samples from both cows and blood samples from case #12-029 and two additional cows were submitted to PPRL for chemical analysis. Liver and blood samples were analyzed for pyrrolizidine alkaloid metabolites (pyrroles) by the method published in Brown et al. (2016) and were detected in all samples, confirming that fiddleneck was likely the primary cause of the poisoning and death losses reported.

Phytochemical Analysis

Previous chemical analyses of the fiddleneck plants from this Arizona case were compared with putatively non-toxic samples from Washington State (Colegate et al. 2013). These fiddleneck species contained the epimers lycopsamine (1) and intermedine (2) (figure 6) mainly present as their *N*-oxides. Minor components were the 7- and 13-acetyl derivatives of 1 and 2 (3 and 4, and 5 and 6, respectively) and the 7,13-diacetyl derivatives of 1 and 2 (7 and 8, respectively), and occasionally a C7 isomer of lycopsamine, tessellatine. As seen in other DHPA-containing plants, there were inconsistent *Amsinckia* alkaloid profiles between specimens purported to be of the same species, population, and phenotype (Molyneux and Johnson 1984). This inconsistency could be due to natural variation, variable degradation due to difference in collection and storage, or taxonomically misassigned specimens. The concentration of total DHPAs (and their *N*-oxides) varied from 1-4-000 µg/g plant leaf material (i.e. 0.0001-0.4% w/dw). As previously

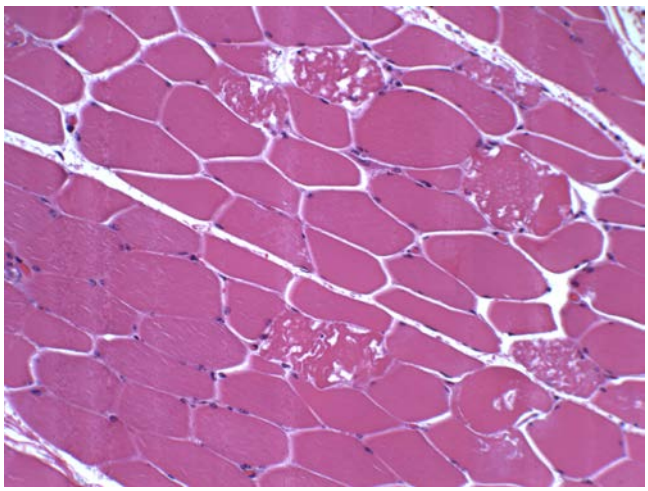


Figure 4. Skeletal muscle from cow 12-029. Notice the focal myofiber swelling hypereosinophilia with coagulation of sarcomere proteins.

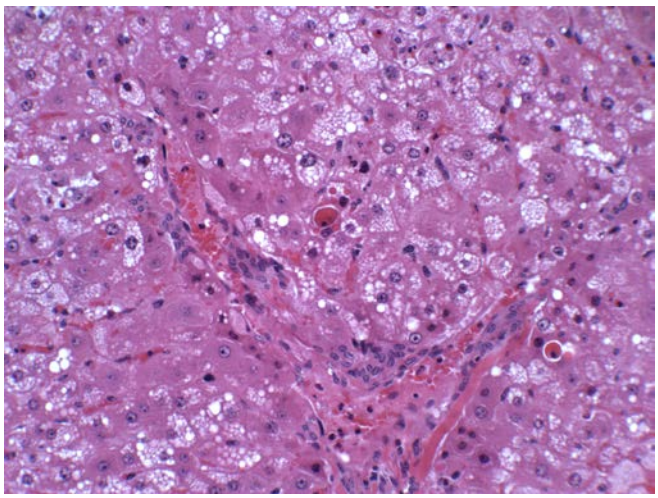
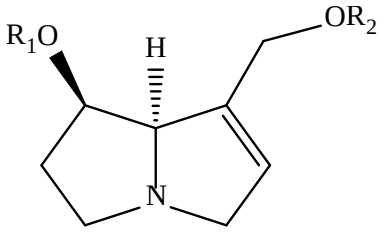
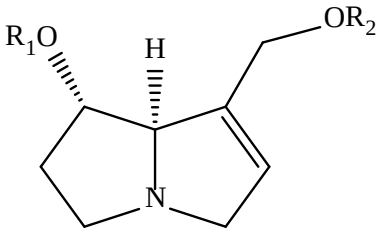


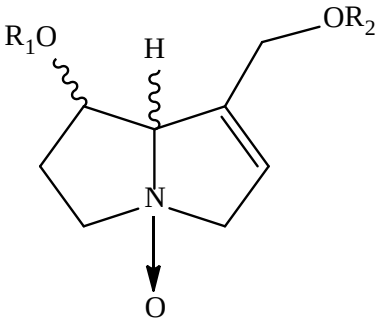
Figure 5. Liver of cow 12-049. Notice the marked hepatocellular swelling and vacuolation with foci of hepatocellular necrosis with residual cellular debris and apoptotic bodies. There is biliary and oval cell proliferation with mild periportal fibrosis with monocytic inflammation.



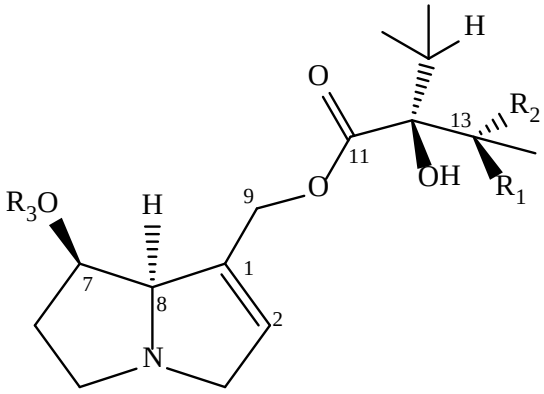
general retronecine



general heliotridine



general dehydropyrrolizidine-*N*-oxide



Compound	R_1	R_2	R_3
lycopsamine (**1**)	H	OH	H
intermedine (**2**)	OH	H	H
7-acetyllycopsamine (**3**)	H	OH	$COCH_3$
13-acetyllycopsamine (**4**)	H	$O(CO)CH_3$	H
7-acetylintermedine (**5**)	OH	H	$COCH_3$
13-acetylintermedine (**6**)	$O(CO)CH_3$	H	H
7,13-diacetyllcopsamine (**7**)	H	$O(CO)CH_3$	$COCH_3$
7,13-diacetyllcopsamine (**8**)	$O(CO)CH_3$	H	$COCH_3$

Figure 6. Chemical **s**tructures of the retronecine base, its N-oxide, and associated dehydropyrrolizidine alkaloids.

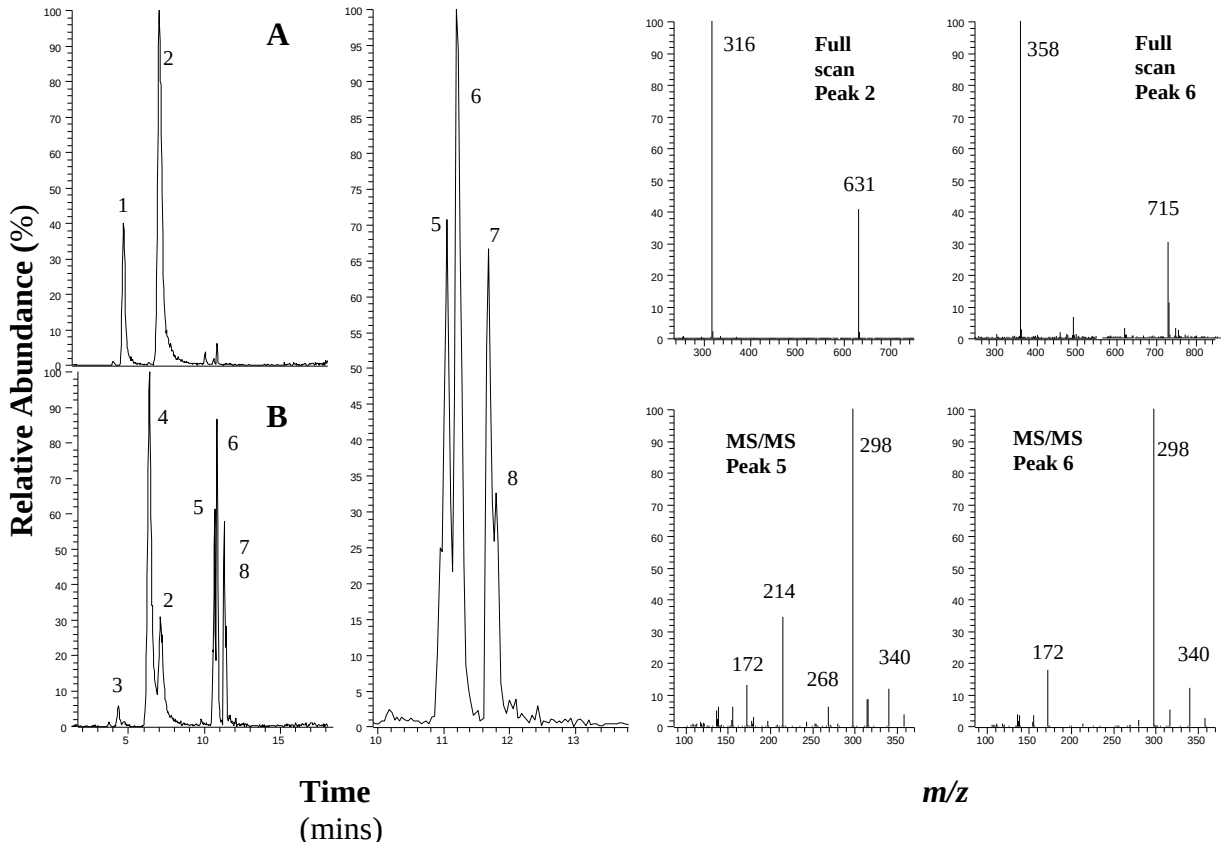


Figure 7. Reversed phase HPLC-esi(+)MS base ion chromatograms of crude methanol extracts of *Amsinckia intermedia* collected from (A) site of cattle poisoning in Kingman, AZ, and (B) one of three sites in Washington State with no record of poisoning. Peak 1 is lycopsamine; peak 2 is lycopsamine-*N*-oxide; peak 3 is intermedine; peak 4 is intermedine-*N*-oxide; peaks 5 and 6 are 7-acetyl and 13-acetylintermedine-*N*-oxides, respectively; and peaks 7 and 8 are the 7,13-diacetyl derivatives of intermedine-*N*-oxide and lycopsamine-*N*-oxide, respectively. Structures are shown in figure 6. Also shown are the full scan mass spectra of peaks 2 and 6 showing the protonated molecule and the significant dimer ion indicative of *N*-oxide structure, and the MS/MS spectra of peaks 5 and 6 showing the significant differences diagnostic of a 7-acetyl and a 13-acetyl derivative at peaks 5 and 6, respectively. *m/z*, mass to charge ratio; MS/MS, tandem mass spectrometry.

reported, *A. intermedia* seeds did not contain DHPAs (Colegate et al. 2013, Johnson et al. 1985). This Arizona *A. intermedia* differed from the other collections as it is primarily the *N*-oxide of lycopsamine (1) with a very minor amount of the 7-acetyl derivative (figures 7 and 8). When compared with other *A. intermedia* collections, the Kingman, AZ samples were about 3-4 times higher than other *A. intermedia* collections; at the time of the poisoning, DHPA concentrations were about 2% DM (Colegate et al. 2013).

Chemical analysis of the burrow weed by high pressure liquid chromatography (HPLC) using the method reported by Lee et al. (2009) showed that the plant material contained 0.32 µg tremetone/mg plant and 0.93 µg dehydrotremetone/mg plant. These concentrations are comparable to those reported (Lee et al. 2015) for other *Isocoma* spp. in the southwestern United States. Historically, tremetone has been reported to be the toxin in *Isocoma* spp., but it has never been experimentally proven. Recent research has demonstrated that the relative toxicity of tremetone-containing plants (*Isocoma* spp. and *Ageratina altissima* known as white snakeroot) does not correlate with tremetone concentrations in the plant (Davis et al. 2016); nor do extracts containing tremetone produce toxicosis (Davis et al. 2015), which has raised some doubt about tremetone being the toxin as previously thought. However, it should be noted that every plant population that has been dosed at PPRL and reported (Davis et al. 2013a,b, 2016), as well as several other species that have been dosed and not yet reported, have contained a significant amount of tremetone.

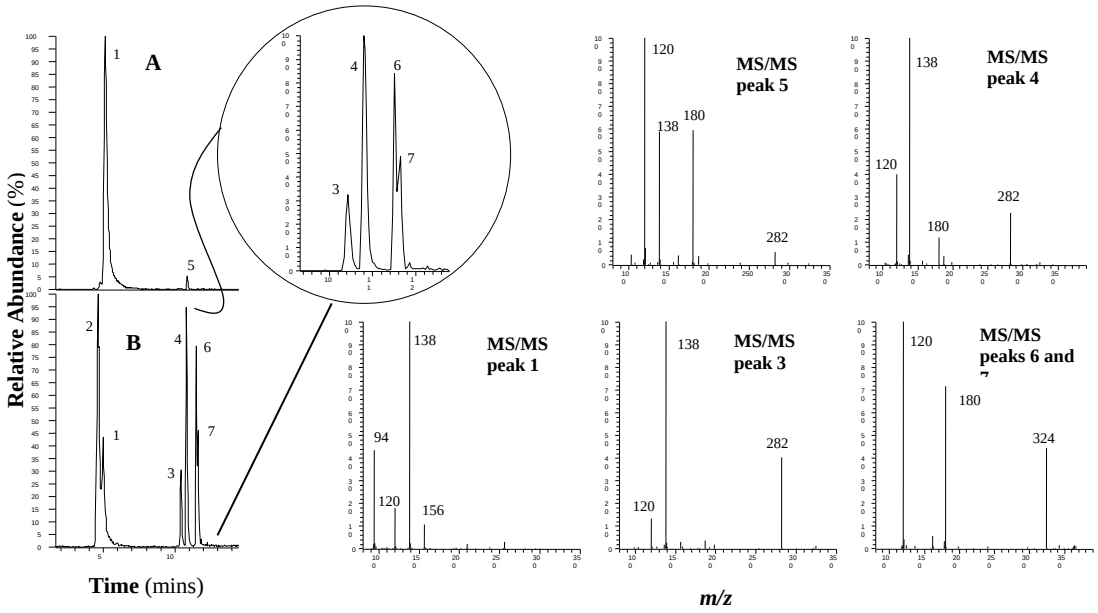


Figure 8. Reversed phase HPLC-esi(+)MS base ion chromatograms of the reduced crude methanol extracts of *Amsinckia intermedia* collected from (A) site of cattle poisoning in Kingman, AZ, and (B) one of three sites in Washington State with no record of poisoning. Peak 1 is lycopsamine; peak 2 is intermedine; peaks 3 and 4 are 7-acetyl and 13-acetylintermedine, respectively; peak 5 is 7-acetyllycopsamine; and peaks 6 and 7 are the 7,13-diacetyl derivatives of intermedine and lycopsamine, respectively. Structures are shown in figure 6. Also shown are the MS/MS spectra of selected peaks. *m/z*, mass to charge ratio; MS/MS, tandem mass spectrometry.

Discussion

The clinical findings, field investigation, and gross and microscopic lesions and chemistry indicate that these cattle were poisoned with both fiddleneck and burrow weed. The DHPA-induced liver disease was the most severe and likely the primary cause of death; however, there are also significant clinical signs and lesions indicative of burrow weed poisoning. As both toxins likely deplete cellular anti-oxidative systems, it is likely that the burrow weed potentiated DHPA toxicity by depleting oxidative preserves such as glutathione and superoxide dismutase. More work is needed to determine if this is the case and better define current management to avoid such potentiated intoxication.

This is the first report of *A. intermedia* poisoning in free-ranging cattle. Previous poisonings were commonly associated with seeds and contaminated grain (McCulloch 1940a,b; Kennedy 1957). Those early reports indicated that seeds contained 0.5% total DHPA and were much more toxic than other plant parts (Fowler and Schoenthal 1967). Neither the *A. intermedia* assay done in 1985 nor that done in 2013 detected DHPAs in the seeds, and the other plant parts had higher but variable DHPA concentrations (Johnson et al. 1985, Colegate et al. 2013). The lack of seed toxicity lends question to earlier studies and the etiology of those previously described diseases such as "walking disease" or "Walla Walla hard liver disease." There are several additional references to *A. intermedia*-contaminated hay poisoning dairy calves. However, this was also a combined intoxication as the hay was reported to contain about 10% *A. intermedia* and 10% *Senecio vulgaris* (Fowler 1968). As *S. vulgaris* contains several DHPAs that are much more toxic than the *Amsinckia* toxins, the *Senecio* probably played a larger role in those poisonings. Both intermedine and lycopsamine have reported LD_{50}s of about 1,500 mg/kg in rats (Fowler and Schoenthal 1967), although cows are more sensitive than rats. Also, these studies were done using a single oral dose, and the toxicity is likely to be higher in more susceptible species such as horses or cattle exposed for longer durations. The *A. intermedia* populations thus far studied have total DHPA concentrations of around 0.5%. Therefore, if poisoning in cattle were to occur, fiddleneck would need to be the bulk of the diet in cattle, and this appeared to be the situation in this case. Furthermore, it may be that *A. intermedia* is only toxic under certain conditions. Similar sporadic poisoning

has been attributed to other DHPA-containing plants. *Echium plantagineum* is known as "Salvation Jane" in parts of Australia and is the only available forage for livestock in some areas. In other areas, it is known as "Patterson's curse" due to its poisoning of animals. More work is needed to determine if *A. intermedia* similarly has two faces. Many different components contribute to toxicity, and those components need to be identified and understood before the underlying risk can be determined and strategies to avoid poisoning can be devised. In this report, we propose yet another factor as combined intoxications seem to be important. Concurrent burrow weed poisoning seems likely to have potentiated fiddleneck poisoning in these cattle.

Conclusion and Implications

The evidence and subsequent research confirmed that the cattle poisoning in this case was caused by *Amsinckia intermedia*-induced liver failure, potentiated by *Isocoma acradenia*-induced myocardial and skeletal muscle necrosis. This is the first documented incident of *A. intermedia* poisoning in free-ranging cattle. Certainly, the underlying cause was poor range conditions and lack of adequate quality forage for these cattle. This was partially due to drought conditions, but cattle cannot be expected to thrive or even survive under such range conditions without a poor outcome. Too often this is a common pattern in large livestock losses from poisonous plants. Having said this, and notwithstanding any differences in rangeland character or grazing/livestock management practices, the quantitative difference or the difference in profiles of the DHPA suites for the Arizona and Washington State plant collections (figure 9) could both play a part in apparent differential toxicity. Additional research on *Amsinckia* toxicoses is needed, but perhaps more importantly is the lack of research on the effects of ingestion of multiple plants and the combined intoxications. More research is definitely needed in this area to better define risk, avoid poisoning, and ensure animal health.

Acknowledgments

The authors appreciate the technical support and editorial contributions from Terrie Wierenga at the USDA-ARS Poisonous Plant Research Laboratory.

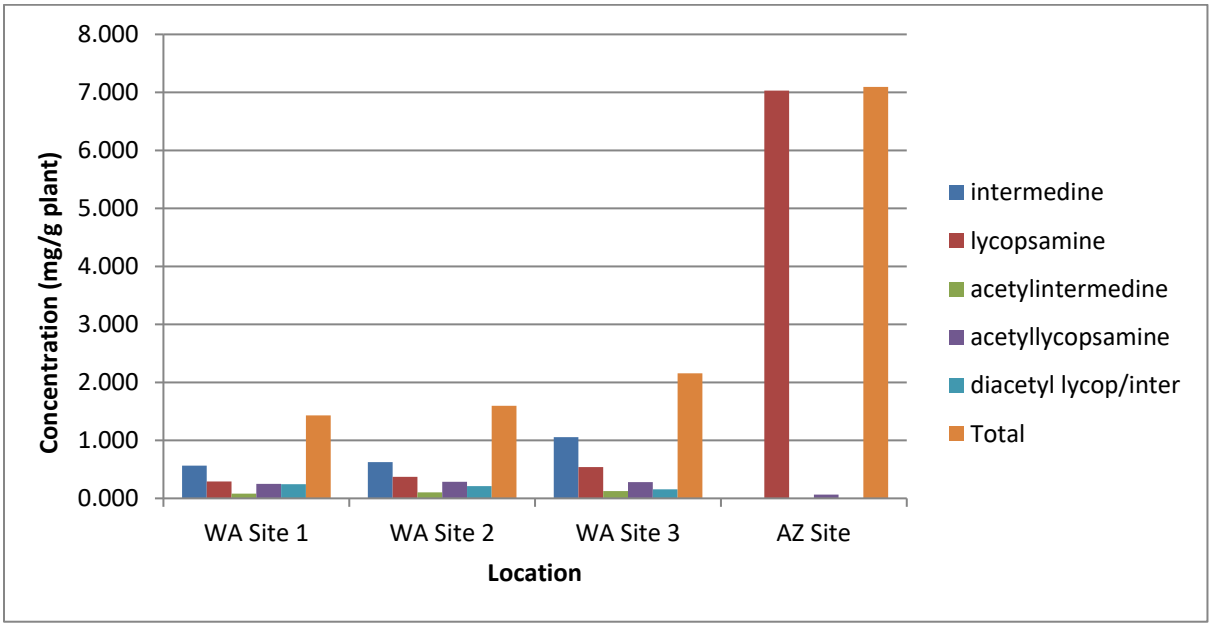


Figure 9. Relative concentrations of dehydropyrrolizidine alkaloids from extracts of *Amsinckia intermedia* collected from three sites in Washington State (WA), USA, where livestock poisonings have not been reported, and the range in Arizona (AZ), USA, where the cattle poisoning in this case occurred. The phenological stage of plant development from the Washington State plants and the Arizona plants was not the same.

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Amsinckia Lehmann (Boraginaceae): A Summary Taxonomic Review

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Abstract

Amsinckia spp., commonly referred to as fiddleneck because of their scorpioid cymes, are toxic plants that cause livestock poisoning from liver disease and associated secondary conditions. A case reporting the death of 15 cows caused primarily by ingestion of *Amsinckia intermedia* published in this issue as a companion paper provided the impetus for this summary taxonomic review. Therefore, an overview of *Amsinckia* as known in the western United States is provided. Because of the confusion and mis-information about the classification of the *Amsinckia* species in general, this review of the taxonomy of this genus is timely and a contribution to the accompanying case report. For this review we have restricted the hundreds of segregate *Amsinckia* species provided in the literature historically to eleven species and an additional half dozen infra specific taxa.

Key words: *Amsinckia*, Boraginaceae, fiddleneck, livestock, poisoning, pyrrolizidine alkaloids, taxonomy

Introduction

Most if not all *Amsinckia* species contain hepatotoxic dehydropyrrolizidine alkaloids (DHPA; Colegate et al., 2013), although poisoning has only been reported from four species *A. lycopsoides*, Lehm, *A. menziesii*, Lehm, *A. intermedia*, Fisch and C.A. Mey, and *A. tessellata*, Gray (Kingsbury, 1964; Burrows and Tyrl, 2013). A recent poisoning case reporting deaths of 15 cows from a herd of 150 ingesting *Amsinckia intermedia* is published as a companion paper in this issue (Panter et al. 2017). Because of this case report and the mis-information in the literature concerning the taxonomic classification of the *Amsinckia* genus, we determined that a summary review of the taxonomy of the genus would also be beneficial to include in this issue subsequent to the case report of poisoning. Therefore, we provide a detailed taxonomic summary review of the genus.

The *Amsinckia* genus is represented in western North America, endemic in California (Kelley and Ganders, 2012) but is also present in South America (Bolivia, Chile, Patagonia) and is widely distributed elsewhere in North America and other countries in the world through introduction (Macbride, 1917). California is most certainly the center of distribution for the species in North America (Ganders, 1993; Kelley and Ganders, 2012), and supports a series of relatively narrowly restricted endemics including *douglasiana, eastwoodiae, grandiflora, lunaris,* and *vernicosa*, some of which are considered as rare and one of them, *grandiflora*, regarded as endangered, probably due to interdiction of its habitat by appurtenances of humanity. Distribution of weedy members of the genus

subsequent to arrival of colonists in the American West have allowed some species to expand far beyond their original ranges. While there are no truly adaptive features that allow ease of transport, the four nutlets (actually schizocarps) are of a size to allow limited dispersion by both birds and rodents, and transport through human activity. North American representatives of the genus *Amsinckia*, mainly *lycopsoides* and *menziesii*, are reported as waifs along roadways or railroad rights of way for all states except for South Dakota, Kansas, Minnesota, Iowa, Arkansas, Louisiana, Alabama, Florida, West Virginia, New Jersey, Maryland, Delaware, and Vermont, and are likely to be found in those states ultimately (Welsh, unpublished data). They are also reported from the prairie provinces of Canada—Alberta, Saskatchewan, and Manitoba—and the Yukon and British Columbia (Welsh, unpublished data). Some species are traditionally confined to smaller regions where natural features (isolated mountain ranges, dunes, or islands) have potentially precluded ease of distribution. Certainly the opening of the American West to occupation by humans and their concomitant clearing of land for agriculture and inadvertent introduction of pestiferous weeds have increased propensity of at least some of the *Amsinckia* species to increase their ranges.

Seeds (the nutlet schizocarps) are often about the size of grains of wheat and can easily be harvested and transported with cereal grains in commerce, either with the cereal grains per se or with forage for livestock and feed for poultry. The plants can be included in bales of hay cut from dry-land growing areas, and can thereby be transported over large distances. Possibly through such transport as feed for horses, they were introduced into Alaska, Yukon, and adjacent northern British Columbia (*A. menziesii*, *A. lycopsoides*, and possibly *A. spectabilis*, (Welsh and Moore, 1968) dating from the late 1890s gold rush era and have persisted since that time. Not only were they introduced in the eastern states, but in Europe, and in Australia, where *A. intermedia* is reported to be a common weed in agricultural lands in New South Wales, Victoria, and Queensland. Macbride (1917) cites a report of one species being prevalent as a weed in grain fields in eastern Washington. The Manual of Vascular Plants of Texas (Correll and Johnston, 1970) includes two species represented in collections by specimens per se, i.e., *A. micrantha* Suksdorf and *A. intermedia* Fischer & Meyer (which they call “Rancher’s fireweed), and *A. lycopsoides* Lehmann, based on a report but of which no specimens had been examined. Whether any were indigenous in Texas or represent man-related introductions is not specified. Stevens (1950) reports two species, *A. idahoensis* (= *menziesii*) and *A. menziesii*, the former taken along railroad tracks at Pembina in 1912 and Rugby in 1918 , and the latter at Fargo, North Dakota in 1942. Kaul (1986) indicates that both *A. lycopsoides* and *A. intermedia* are both seldom collected and only occur as waifs in the Great Plains.

Despite having attractive yellow or orange flowers arranged along the axis of a scorpioid cyme that uncoils as the plants mature, the plants are not highly regarded as ornamentals, “but often included in wild flower seed mixtures” (Hitchcock, et al., 1959). Ganders (1993) nevertheless gives an obscure reference to their use as ornamentals, when for *A. grandiflora* is the notification, “In cult”. The scorpioid cyme, uncoiling from the tip (figure 1), is indeed architecturally striking. One need only look at photographs of the various species available on the internet to see how beautiful the flowers can be, but the plants per se offer little in the way of stature or other features to be considered other than drab by comparison with other cultivated ornamentals. Herbarium specimens are anything but beautiful!

Figure 1. *Amsinckia* line drawing and photo of the flower head showing the scorpioid cymes. Drawing by Holly Hyer, Utah State University, photo by K.E. Panter.

Number of Entities

Over the years, the number of species (including infraspecific taxa) thought to exist varies widely. Gray (1878) cited only six species for North America, and two varieties. Macbride (1917), in an attempt to provide an overview of the genus treated some 23 species, six described by him therein as new or previously unmentioned. He noted that “. . . it must be conceded that characters which admit of clean-cut statements are all too few.” The notice by Macbride understates the case. The lack of consistent diagnostic characteristics in

the genus has plagued botanists from the beginning. Intermountain Flora (Cronquist et al., 1984) indicates that there are about 15 species native to western North America and southern South America. Correll and Johnston (1970) note therein that the genus consists of "about 50 species of considerable technical difficulty which centers in western North America." Wilhelm Nicolas Suksdorf (1850–1932) named 209 species of *Amsinckia* based on minutia of morphology (Suksdorf, 1931), not only taxa per se. He named specimens, not species, and in spite of the great number so named, only uncommonly have any of his *Amsinckia* "species" gained acceptance in modern interpretations of floras. Instead they clog the synonymy of the relatively small number of species known for the genus. A rather detailed biographical account of Suksdorf by Rhoda M. Love is reprinted in Botanical Electronic News (No. 385; December 2007) based on *Wilhelm Nikolaus Suksdorf (1850–1932) Pioneer Botanist of the Pacific Northwest* (Pacific Northwest Quarterly 89(4), 1998).

Members of the genus are made up of self-pollinated and cross-pollinated individuals and strains, and thus lead to formation of both self-perpetuating and hybrid groupings. This can be confusing when attempting to classify this complex genus. In writings on the role of heterochrony in flower development and evolution of self-fertilization of *Amsinckias*, Li and Johnston (2001; 2010) studied 26 flower traits under natural conditions on 3 clades of *Amsinckia*. The evolutionary changes they reported in flower morphology alone is complex and confusing, thus, when all of the plant traits are combined the temptation for taxonomists to expand the genus using minute characteristics is tantalizing. Attempts to make sense out of the resulting genetic interaction have plagued taxonomists from the start, and Suksdorf was apparently baffled by them. What appeared at first to be a rather small grouping of species took on a troublesome system of genetic and phenotypic variability that led Suksdorf to classify species based on an endless number of minor morphological phases (209 species). Suksdorf's herbarium and writings are at Washington State University. Brand in Report. Sp. Nov. Regni Veg. (volumes 20, 25, and 26; 1925, 1928, and 1929, respectively) named several critical species in the genus, basing his observation on specimens from North America then in European herbaria. None of his half dozen proposals stand at taxonomic rank in contemporary literature. He was apparently baffled by the variation available in this remarkable genus, which was obviously in evolutionary transition when first encountered by botanists, and which is undergoing additional evolutionary changes where it has encountered habitats not available to it prior to the advent of western civilization.

Morphological Characteristics

Members of the genus attracted the early attention of Botanists as indicated by the naming and classifying of the genus *Amsinckia* by Lehmann (1831), and like many other genera of plants the morphological features are sufficiently plastic and intergrading that few, if any are diagnostic when used solo, and even in combination are difficult to apply. Except for the three taxa with smooth and glossy nutlets, the remainder have nutlets whose surfaces are variously sculpted (some with surface resembling road pavement). Thus, most of the nutlet characteristics used to distinguish entities in the genus are subject to interpretation, and variation within them leaves the investigator often baffled. The sculpturing of the nutlets is not the only source of bafflement i.e. other vegetative features such as degree of hispidity and the positional placement of epidermal trichomes from stem base to apex or less so below than above, or the existence of pilosity in addition to hispidity and its placement, or differences in trichome abundance between lower leaf surface and the upper, where sometimes the trichomes are present only along the midrib, to name a few. Pilosity beneath the hispid hairs on stems (upper only) or on calyx lobes, is also subject to interpretation. There are few or no absolute diagnostic criteria presented that will serve to distinguish one taxon from all others, including heterostyly versus homostyly, features often shared within a given entity.

Staminal filament attachment can be and is often variable, not only in the genus as a whole but in the individual species, the anthers thus produced below, adjacent to, or above the tip of the style, and in some cases, two of the filaments are short and the other three are elongated. Number of veins in the lower portion of the corolla below the staminal attachments, 10 or 20 as the case may be, appear to have taxonomic significance, but is difficult to determine in pressed material. Coalescence of calyx lobes, resulting in two to four apparent lobes (by fusion into sets) apparently has value as a diagnostic feature, but again is difficult to discern, at least in pressed materials. The lobes, whether coalesced, or distinct are hidden in the accompanying mass of elongate, sharp trichomes.

Corolla limb width varies within some species, sometimes not forming a continuum, but as distinctive corolla-size phases. What one would hope to place in a small-flowered taxon often finds placement in another

due to divergent nutlet characteristics, characteristics that are judged to override corolla sizes for taxonomic placement. Kelley and Ganders (2012) for example treats *gloriosa* as a variety of *tessellata*, separating them on corolla size, 12–16 mm long and limb 6–10 mm wide for var. *gloriosa*, and 8–12 mm long and limb 2–6 mm wide for var. *tessellata*. He treated *intermedia* as a variety of *menziesii* again based on flower size, i.e., corolla 4–7 mm long and limb 2–3 mm wide and yellow for var. *menziesii*, and corolla 7–11 mm long and limb 4–10 mm wide and more or less orange for var. *intermedia*.

Plant stature has been used in combination with other features (Ganders, 1993) to distinguish var. *spectabilis* from var. *microcarpa*. They were separated by him on the basis of stems erect, calyx lobes distinct, nutlet 1–1.5 mm long; flowers heterostylous, for var. *microcarpa*, and stems generally decumbent; calyx lobes, 2 or 3 of them half coalesced; nutlet 1.5–2 mm long; flowers heterostylous or not. It is to be noted that in the above cases nutlet size and corolla measurements form a continuum.

Evident lack of genetic incompatibility is recognized by the presence of apparent or actual hybrids between *lycopsoides* and *menziesii*, between vars. *spectabilis* and *microcarpa*, and between vars. *vernicosa* and *furcata*. Perhaps evidence of intermediacy is obscured, at least sometimes, by lack of actual morphological differentiation.

Still, as pointed out by Hitchcock et al. (1959), "Among our [Northwestern American] species *A. lycopsoides*, *A. spectabilis*, and *A. tessellata* are sharply limited and technically well marked, distinguishable without difficulty." They also noted that except for *A. spectabilis* "our species are all weedy." To the list of weedy species can be added *A. menziesii*.

Chromosome numbers and position of stamens with regard to position of stylar tip were determined for eight species by Ray and Chisaki (1957). Where anther placement was equal to the style in position they are regarded as homostylic, but where their position is either above or below the stylar position they are regarded as heterostylic. Determinations by Ray and Chisaki (1957) are as follows: *douglasiana* n = 6 (heterostylic); *furcata* n= 7 (heterostylic and homostylic); *gloriosa* -12 (homostylic); *grandiflora* 6 (heterostylic); *lunaris* 4 (heterostylic and homostylic); *spectabilis* 5 (heterostylic and homostylic); *tessellata* 12 (homostylic); and *vernicosa* 7 (homostylic). Additional chromosome numbers taken from literature are cited following the species descriptions below.

Ray and Chisaki (1957) note, "In almost every one of the heterostylic species of *Amsinckia*, this heterostyly appears to have been in various ways replaced by homostyly, which subsequently has led to the appearance of small-flowered, clearly self-pollinated forms." Thus, their phylogenetic chart reflects the heterostylic, large-flowered *furcata* to homostylic large-flowered *furcata* to small-flowered self-pollinated *vernicosa* (and the widely disjunct *carinata* as well). *A. tessellata*, both small-flowered and homostylic terminates the line of heterostylic *grandiflora* and *douglasiana* through *gloriosa*. *A. lunaris* displays all heterostylic phases and a small-flowered homostylic phase as well. They show large-flowered homostylic *eastwoodiae* as basal to homostylic smaller flowered *intermedia* and *lycopsoides*, and small-flowered *A. menziesii*.

Coalescence of sepals versus sepals distinct appears to be significant, and has been utilized as diagnostic in separation of the *Amsinckia* species into two rather distinctive groups. However, in at least some instances the first to open flowers on the cyme are reported to have separate sepals even in those entities with all later flowers having coalesced ones. Too, it is difficult to distinguish calyx characteristics in pressed material where that feature is obscured by the thatch of pungent trichomes. The feature of coalesced sepals is most easily discerned in mature plants wherein the cyme has elongated into a unilateral spike and nutlets have been shed or about to be shed. The sepals are then displayed widely separated, and one can check the lobes to determine if two or more main veins are present, or if there is a notch at the tip of the "sepal."

Taxonomy

Suksdorf (1927) published a paper Washingtonische Pflanzen IV in which he wrote a summary treatment for *Amsinckia* in the flora of Washington State. Therein, he recognized three sections (his Abteilung), which were treated as such in the 1931 summary revision, they are *Muricatae* (p. 49), *Tessellatae* (p. 102), and *Vernicosae* (p. 112). Later in his 1931 treatment he recognized a fourth section, i.e., *Microcarpae* (p. 94), whose species had been included in *Muricatae* previously. Largest of the sections is *Muricatae*, in which are included most of the hundred plus species he described as new, and also most of those described by others previously. Smallest of the sections is *Vernicosae*. The sections have been recognized by other workers (see reference section) with some modifications and additions. The following sectional descriptions are translated from the Suksdorf 1927 treatment.

Section *Muricatae* – Calyx lobes five, distinct; corolla tube 10-nerved; nutlets 1.5–3.5 mm long, 4-

angled-ovoid, the dorsal ridge clearly distinct, seldom almost lacking, the transverse ridges at the base of the nutlet approach the hilum at its highest point, or it reaches it not fully, so that a small gap remains (Suksdorf, l.c.;49). In this section (actually Division according to Suksdorf) are included some 155 Suksdorfian species, most of them applicable to *A. intermedia*.

Section *Microcarpae* – [previously, in Suksdorf (1927), included in Muricatae]. Calyx lobes five, or mostly 2 or 3 lobes with each other connate; corolla tube 10-nerved; nutlets 1.5–2.5 mm long, 4-angled-ovoid, the dorsal ridged seldom lacking, the transverse ridges touching the hilum on its broadest place or in the center and forming therewith often a right-angled cross.

Included here were 26 Suksdorfian species assignable to *A. spectabilis* and one to *A. lycopsoides*.

Section *Tessellatae* – Calyx mostly 3-lobed, whose broad lobes are 2-toothed, or the calyx lobes are all distinct in the earliest flowers; corolla tube 20-nerved below the stamens (though seldom the axillary nerves can be lacking); nutlets 2.5–4.5 mm long, the back low to flat, the margins 2-angled, straight, the transverse ridges touching the hilum on its broadest place.

Included here are *A. douglasiana* with two segregates, *A. gloriosa* with five segregates, and *A. tessellata* with 27 segregates, three of them not from the United States.

Section *Vernicosae* – Calyx mostly 3-lobed; corolla tube below the stamens 20-nerved; nutlets 2.5–5 mm long, straight, smooth and shiny, 3-angled, the angle sharp; hilum small or entirely hidden; transverse ridges are seldom recognizable.

Included in this section are *A. vernicosa* (n=7), *A. carinata*, and *A. grandiflora* (n=6) and three segregates. However, Ray & Chisaki (1957), did not recognize the Section *Vernicosae*, but placed it, with its small-flowers derived from *furcata*, within Section *Tessellatae* Suksdorf, whose other alliances included *A. grandiflora* (n=6), leading through *douglasiana* (n=6) to *gloriosa* (n=12) with moderate-sized flowers, and ultimately to small-flowered *tessellata* (n=12), along one branch; and to Section *Microcarpae* Suksdorf, including both large- and small-flowered phases of *A. spectabilis* (n=5), but whose lateral branch became a newly proposed Section *Disjunctae* (Ray & Chisaki, 1957) including the solitary species *A. lunaris* (n=4) with both large- and small-sized flowers, and a questionable tie-in with Section *Muricatae* (Suksdorf, 1927), including large-flowered *eastwoodiae* (n=12), to moderately sized flowers of *intermedia* (n=15, 18, 19) and *lycopsoides* (n=15) and finally to small-flowered *menziesii* (n=8, 13, 17) of *A. menziesii* (Lehmann) (Nelson and Macbride, 1916). Suksdorf (1927) states that he was forced to omit this species because he did not know it, and further that Gray had never named it. However, in his 1927 paper, Suksdorf keyed it adjacent to *A. lycopsoides* in his Abteilung *Tessellatae*.

Sonoran Desert and Baja California distributions of *Amsinckia* – Both Shreve and Wiggins (1964), Vegetation and Flora of the Sonoran Desert and Wiggins (1980), Flora of Baja California treat species of the genus *Amsinckia* that are widespread elsewhere in North America, *i.e.*, *A. intermedia* and *A. tessellata* in Shreve and Wiggins plus *A. menziesii* and *A. spectabilis* in Wiggins. Neither of those treatments cite synonyms, except indirectly. Certainly none of those plants named on the basis of type material from either Baja or the Sonoran Desert are included at taxonomic rank. Examples named by Suksdorf (1931) include *A. nesophila* Suksdorf, Werenda 1: 93. 1931; Type: "Unterkalifornien: Cedros Island, T. S. Brandegee s.n., 3 April 1897, holotype UC [very likely= *intermedia*]; *A. orcuttii* Suksdorf 1108, "Unterkalifornien: Japa, C. R. Orcutt 1108, 4 July 1884, holotype UC, first cited [very likely *A. tessellata*]; and *A. inepta* Macbride, Contr. Gray Herb. 59: 14. 1917; Type: Lower California, San Martin Island, Anthony 217, sans date, holotype GH, related to *A. eastwoodiae*.

Amsinckia Taxonomy–Revised

***Amsinckia* Lehmann** Index Seminum (Hamburg) 3, 7, 1831 (nom. cons.)

Annual, pungent-bristly, herbaceous plants; stems erect or decumbent with spreading branches, leafy; leaves alternate, linear to ovate, usually veinless; racemes usually ebracteate; calyx cut to base into erect lanceolate or oblong lobes; corolla tubular or salverform or funnelform, heterostyled or not, yellow or orange, tube cylindrical, glabrous, unappendaged; lobes spreading, rounded, imbricate; stamens included, affixed in the tube; filaments very short to elongate; anthers oblong; style obtuse, filiform, included; stigma capitate, emarginate; ovules 4; cotyledons 2-parted; nutlets 4, erect, angulate-ovoid, smooth or rough, unmargined, strongly keeled ventrally; gynobase pyramidal, ca half the height of the nutlet (n = 4, 6, 8, 13, 15, 17, 19).

1. Leaves often erose-denticulate; calyx with two sepals usually partly united; plants maritime or of inland dunes ..... *A. spectabilis*
– Leaves entire; sepals either reduced in number (3 or 4 by lateral coalescence) or five and distinct; plants seldom maritime 2
2(1). Calyx lobes 2–4 by coalescence; corolla tube 20-veined near base; nutlet surface smooth, cobble-stone-like, or round-tubercled 3

– Calyx lobes 5; corolla tube 10-veined near the base; nutlet surface generally sharp-tubercled, often ridged 7
3(2). Nutlets smooth and shining 4
– Nutlets variously roughened, not shining 6
4(3). Scar of nutlet, ca 1 mm plus long, borne on the crest of the ventral keel; corolla tube 10-veined below attachment of stamens; plants known only from Malheur Co., Oregon ..... *A. carinata*
– Scar of nutlet either obscured by ventral groove, and nearly basal or conspicuous and nearly median; corolla tube 20-veined below attachment of the stamens; plants of western Mohave Desert and Coast and Inner Coast ranges, California….5
5(4). Leaves coarsely and densely pustulate above, sparsely so beneath, hispid-ciliate; scar of nutlet poorly developed, sublineate or forked, nearly basal ....*A. vernicosa*
– Leaves finely and densely pustulate, hirsute above and beneath; scar of nutlet conspicuous, lance-ovate, nearly median ..... *A. grandiflora*
6(3). Flowers heterostylic, yellow-orange; calyx densely rusty-hairy and bristly nutlets tessellate..... .....*A. douglasiana*
– Flowers homostylic, yellow to yellow-orange; calyx and nutlets various 7
7(6). Corolla limb 2–4 mm broad, much surpassing the calyx; calyx thinly white-hirsute.....*A. tessellata*
– Corolla limb 8–14 mm broad, also much surpassing the calyx, its tube flaring upward..... *A. gloriosa*
8(2). Corolla throat nearly closed by well-developed, hairy fornices; stamens borne below the middle of the corolla tube .....*A. lycopsoides*
– Corolla throat not closed by fornices; stamens inserted above the middle of the corolla tube..... 9
9(8). Corolla somewhat asymmetric, the limb with 2 red-orange marks, the tube bent ..... *A. lunaris*
– Corolla limb not marked, the tube straight 10
10(9). Stem spreading-hispid and also evidently puberulent or strigose with shorter, softer, more or less retrorse hairs; leaf pubescence tending to be ascending, not widely spreading.....*A. retrorsa*
– Stem spreading-hispid, almost or quite without any shorter, softer pubescence below the inflor-escence 11
11(10). Corolla orange yellow, 7–20 mm long, well exserted beyond the calyx; plants usually green; stems hirsute-bristly, but with few or no fine appressed hairs ..... *A. intermedia*
Corolla pale yellow, 4–7 mm long, little or not at all exserted beyond the calyx lobe; leaves pubescent with appressed or ascending hairs ...*A. menziesii*

***Amsinckia carinata* Nelson & Macbride**, Bot. Gaz. 62: 145. 1916. [Type: Oregon, Malheur Co., Malheur Valley near Harper Ranch, at 1100 ft, John B. Leiberg, 2234, 10 June 1896, holotype GH; isotypes, NY, UC; OSU (topotype 2232, 8 June 1896, accession no.155192, photo available on Internet)]

Plants 1-5 dm tall, stems branched above, glabrous to glaucous below, sparsely bristly above; leaves ovate to broadly lanceolate or narrowly oblong, , mostly 1–3 cm long, , with pustulate-based hairs above and below, some of conspicuous pustules and relatively small setae; spikes to 25 cm long in fruit; sepals (by connation) in pairs or threes (similar to *A. tessellata*), corolla yellow to burnt orange, ca 8 mm long, the limb half to about as wide as tube length, the tube 10-veined below staminal attachment; nutlets lance-attenuate in profile, smooth and shiny, 4–7 mm long, ventral keel prominent, the dorsal one low.

Bouldery and gravelly talus slopes, near Harper, in Malheur County, Oregon, where it is known from a small number of localities, within the northern extension of the Basin and Range Province.

Nelson and Macbride (1916;146) in discussion following the description of *A. carinata* note: “It is surprising indeed to find a representative of this alliance so far north. *A. grandiflora* (A. Gray) Kleeberger ex A. Gray, the species which *A. carinata* so closely simulates in habit, has not been collected north of San Francisco or Monterey. The Oregon species does not seem to differ from *A. vernicosa*, except in its fruit; it is only related to *A. grandiflora* in a general way; its nutlets are radically different from both. Mature nutlets of *A. vernicosa* are bright gray, speckled with black, 4–4.5 mm long, sharply triquetrous (like monster buckwheat grains) and with no obvious scar.” The 10-veined condition of the corolla tube below the staminal attachment is apparently diag-nostic for this local endemic from both *A. grandiflora* and *A. vernicosa*.

***Amsinckia douglasiana* A. DC.,** Prodr. 10: 118. 1846. [Type: “In Nova California (Dougl.),” holotype G?, isotype? GH”]

Stems erect, 3–5 dm high, simple or branched below, sparingly hispid below, the bristles more spreading above; leaves lance-linear to lanceolate, sometimes spoon-shaped, appressed-hairy and subcinereous, weakly pustulate; spikes elongating with age, often 10–15 cm long; calyx lobes 2– 4, 1 or 2 pair connate, 6–12mm long in fruit; corolla orange, 12–16 mm long, the limb 6–10 mm wide; nutlets ca 4 mm long, broadly ovoid, flattish and tessellate (like cobblestone) on back, the scar ovate (2n=12).

South coast ranges disjunct from the coast, in Monterrey and San Benito cos. to n. Santa Barbara and w. Kern cos., California. “Coastal hill country from the region of San Francisco Bay to San Diego County” (Macbride 1917;13). “Scattered localities in the Coast Ranges of California from Monterey County to Ventura County” (Ray & Chisaki 1957; 531). Uncommon. Heterostylous.

Type description: “Caule erecto simplicei inferne glabro apice setis pilisque mollibus pubescenti, foliis erectis linearbus acutiusculis pilis basi albotuberuclatis patentibus vel in foliis junioribus adprepssis hispidulis, racemis demum elongatis, calycibus hispidissimi, lacemiis linearibus obtusis, corolla calycis duplo longior, glabra, staminibus in superiori parte corolla inclusis.

Caulis pedalis? Folia radicula ign. Caulina 1–2 poll longa 1–2 lata, superne prasaeritim, pilosa superiore basi latiore et fere lanceolata, raceme simplices vel bifurcatie, florae subsessiles, secundi, calyces pilis subpatentes, juniores fulvi. Corolla 5–6 lin. longa, lutea, apices infundibuliformis, lobes

tamen brevibus, nuculae 2 lin longae pallidae. Char. Gen non dub." (l.c.)

***Amsinckia gloriosa* Eastwood ex Suksdorf,** Werenda 1: 103. 1931. [*A. tessellata* A. Gray var. *gloriosa* (Eastwood ex Suksdorf) Hoover, Vasc. Pl. San Louie Obispo Co., Calif. 245. 1970; Type: California, Cuyama, White Hills, Alice Eastwood, 1 May 1896, zum Teil, holotype CAS]

Stem 4 dm high, hirsute, branched at the ground; cauline leaves lanceolate, thickly hirsute, gray; calyx 6–7 mm long, the wider ones [sepals] the longer, hirsute; corolla 14 (12–16) mm long 6–10 mm wide, orange; anthers generally below stigma; nutlets 3 mm long, deltoid-ovoid, the point tuberculate, the short margin uneven, not flattened, the hilum lanceolate-rhombic (translated from Suksdorf l.c.). 2n = 24.

Abundant in the Coast Ranges from Los Angeles County to Monterey and Alameda and Colusa cos (Ray & Chisaki l.c.), and to Ventura Co., California.

In Abrams (1951) *A. gloriosa* is regarded (along with *A. munzii*) as a synonym of *A. douglasiana*, but in Munz (l.c.), this is distinguished from *A. douglasiana* only in the ratio of flower having a ratio of length of style to length of stamens fairly constant and pollen being tetracolporate, not tricolporate as in that species. In Jepson Manual (l.c.), it is regarded as a variety of *A. tessellata.*, differing in the corolla being 12–16 (not 8–16) mm long, and the limb 6–10 (not 2–6) mm wide, both measurements are obviously continua!

Amsinckia grandiflora (A. Gray) Kleeberger ex A. Gray in Brewer & Watson, Bot. Calif., 1: 525. 1876. or is it **ex Greene** Bot. Man. Bot. San Francisco 262. 1898. [*A. vernicosa* var. *grandiflora* A. Gray, Bot. Calif [W. H. Brewer] 1: 525. 1876; Type: California, Contra Costa Co., at Antioch, Kellogg & Harford s.n., 16 April, 1869, holotype GH.]

Stems erect, 3–6 dm high, hispid with spreading bristles sparingly hispid below, thinly pilose above and the stiffer hairs weak or represented by their pustulate bases only; leaves linear to narrowly ovate, pustulate on both surfaces but the bristles often not developed; spikes elongating with age, often 10–15 cm long; calyx lobes 3 or 4, with rust-colored bristles that often completely conceal the appressed hairs beneath, to ca 12 mm long; corolla orange, 14–18 mm long, the limb 8–10 mm wide, the tube 20-veined below attachment of stamens; nutlets 3–4 mm long, ovoid, smooth and shining, the scar broadly lanceolate, a little below the middle.

Coast Ranges, Monterey and San Benito cos, to n. Santa Barbara Co and w. Kern Co., Calif. Cited as Endangered and presumed extinct near Antioch, Contra Costa Co., California in Jepson Manual (l.c.; 368).

A recovery plan available on the Internet indicates that the present known distribution consists of two small colonies in Corral Hollow, Alameda and San Joaquin counties, and a second population in western San Joaquin County, all south of Antioch, and southeast of Mt. Diablo.

***Amsinckia intermedia* Fischer & Meyer,** Ind. Sem. Hort. Petrop. 2: 25. 1836. [*A. menziesii* var. *intermedia,* (Fischer & C. Meyer) Ganders, Jepson Man. 368. 1993, nomen illeg., sans basionym; Type: California, Bodega Bay, "circa coloniam ruthenorum Ross in portu Bodega," Eschscholtz s.n, holotype LE.] Tarweed fiddleneck

Stems simple or much branched, erect to widely spreading, 3–9 dm tall, sparsely bristly otherwise usually glabrous except for a tomentose pubescence near the base of the spikes; basal and lower cauline leaves to 15 cm long and 1 cm wide, linear or linear-lanceolate to nearly ovate, usually clasping at base and acute at apex, thinly hirsute on both sides with spreading, often pustulate hairs; spikes short or usually elongating in fruit, usually leafy-bracteate at base; calyx lobes linear attenuate, ca 5 cm long in flower, 6–12 mm long in fruit, about half as long as the corolla, rufous-hispid on the back, densely white-hirsute on the margins; corolla orange yellow 8–10 mm long, the limb 3–6 mm wide, the throat open; nutlets 2.5–3 mm long, incurved, grayish, narrowly keeled on the back and sharply rugose with the surface between papillate and muricate; scar shape, position? (n = 17, Windham & Windham 94–14, BRY!; n = 15, 17, 19, Ray & Chisaki 1957; 548; Munz, 1959; Fl. Calif. 589).

Creosote bush, Joshua tree, and other warm desert shrub communities below 1200 m in Box Elder, Davis, Millard, Utah and Washington cos.; Wash., s. to Baja Calif. and e-ward to Ariz., N. Mex., and w. Texas; Parry 68, 1875 ISC!; 30 (xi).

An indication of the difficulty of interpretation of standing for a given proposed entity is to be found in *A. micrantha* Suksdorf, which was cited in synonymy of *A. menziesii* by Ray and Chisaki (l.c.), but was regarded at specific level by Correll & Johnston (l.c.).

The material from north of Washington Co. Utah is apparently somewhat intermediate to *A. tessellata*. Specimens of this species are also reported for Tooele and Rich cos. The plant is known to be poisonous to all classes of domestic livestock, due to pyrrolizidine alkaloids.

However, the seeds are reported in the Internet to be the favorite food of Lawrence's goldfinch in California during the nesting season.

A note in Munz (Calif. Flora; 589) indicates that Suksdorf recognized over 100 segregates! However, Munz recognized only two varieties, as indicated below, but a vastly polymorphic third variety, var. *intermedia*, is automatically understood to exist. And, although *A. intermedia* has been regarded at specific rank for more than seven decades it (they, them) was (were) proposed at varietal rank by Ganders (Jepson Manual; 368) within *A. menziesii*, the proposal lacking citation of the basionym being illegitimate. Ganders does, however, comment that this plant by whatever name hybridizes with *lycopsoides*.

var. ***echinata* (A. Gray) Wiggins, Contr. Dudley Herb. 4: 22. 1950. [***A. echinata* A. Gray, Proc. Amer. Acad. 10: 54. 1874. "Corolla ut videtur flava, tenuis, lin. 3–4 mm longa, fauce vix ampliata; antherae lineari-oblongae, aut fauciales exserte aut infra medium tubi; calyx rufo-hirsutus; nuculae granulato-rugolosae vel submuricatae." Type: "Southeastern part of California, [Fort Mohave] Dr. J. G. Cooper s.n., Feb. 1861," holotype GH?]

Nutlets ovoid, ca 2 mm long, the dorsal keel drawn up into a fragile knife-like edge, the surface rough papillate, growing in Creosote Bush Scrub, in the eastern Mohave Desert.

Kearney & Peebles (1942;760) note that, "*Amsinckia echinata*, based on material from near Fort Mohave (*Cooper*

in 1860), is a form having the tuberculations and the dorsal keel of the nutlets elevated, narrow, and fragile. Such plants are frequent in Arizona and adjacent California, but are connected by many transitions to the forms with less prominently roughened nutlets that are typical of *A. intermedia*," and further, "Suksdorf described various forms of *A. intermedia* as *A. nana*, *A. demissa*, *A. rigida*, *A. arizonica*, and *A. microphylla*, all based on Arizona types" (Johnston, 1953; see also synonyms of *A. intermedia* below).

var. ***eastwoodiae*** **(J. F. Macbride) Jepson & Hoover,** Fl. Calif. (Jepson) 3: 323. 1943. [*A. eastwoodiae* J. F. Macbride, Contr. Gray Herb. 49: 14. 1917; *A. douglasiana* var. *eastwoodiae* (J. F. Macbride) I. M. Johnston, Bull. S. Calif. Acad. Sci. 17: 66. 1918; Type: "California, near Pollasky, Fresno Co, April 11, 1906, Heller, no 8153," holotype GH, isotype CAS]

Corolla deep orange, 14–18 mm long, two- to three-times as long as the calyx, its tube flaring, the limb 8–14 mm wide; nutlet rather large (n=12; Ray & Chisaki 1957;548).

Great Valley from Shasta County south, also South Coast Ranges and as far south as Riverside County California.

A. eastwoodiae is regarded at specific rank in Jepson Manual (1993; 368) and by Ray and (Chisaki 1957; 531), but at varietal rank in Abrams (1951;607), based on corolla 15–20 mm long and deep orange. Ganders (Jepson Manual, 1993; Kelley and Ganders, 2012) keeps *eastwoodiae* at specific rank, but comments that it is "Like large-fld pls of *A. menziesii* var. *intermedia*."

Amsinckia lunaris **J. F. Macbride,** Contr. Gray Herb. 49: 12. 1917. [Type: California, San Mateo Co., grassy bank near San Mateo, on the Half Moon Bay Road, Heller 8555, 23 May 1907, holotype GH.]

Stems simple or much branched, erect to widely spreading, 3–9 dm tall, sparsely bristly, otherwise usually glabrous except for a tomentose pubescence ["ad apicem dense retroroso-strigulosis"] near the base of the spikes; basal and lower cauline leaves linear or linear-lanceolate ["circa 3 mm latis 4 cm longis"] to nearly ovate, usually clasping at base and acute at apex, thinly hirsute on both sides with spreading, often pustulate hairs; spikes short or usually elongating in fruit, usually leafy-bracteate at base; calyx lobes 5 ["4–5 mm longis," or becoming longer at maturity, bristly with gray to rust-colored hairs]; corolla bilateral, red-orange, 7–10 mm long, the limb 5–7 mm wide, with 2 red-orange marks; nutlets 2–4 mm long, ["sublunatis"] tubercled and sometimes ridged (2n = 8).

Scattered localities in the Coast ranges, in the vicinity of San Francisco Bay, and eastward into the great valley of California.

Macbride (l.c.) indicates that this is a segregate of *A. douglasiana*, but "apparently very distinct because of the very pubescent upper leaves and the not at all rugose nutlets.

Munz (1959; 589) notes that this differs from *A. intermedia* in it heterostylic, more or less asymmetric, bilaterally marked flowers the limb with two red-orange marks, and with bent corolla tube.

Two specimens are cited by Suksdorf (l.c.) with the protolog of *A. papillata*, the first cited, i.e., California, Fresno Co., (Julia McDonald) with the notation "kurzgriffelig;" and the second, California, Tulare Co., Pixley, Eastwood s.n., as "langgriffelig," i.e., short-styled and long-styled, respectively.

Amsinckia lycopsoides **Lehman ex Fischer & C. A. Meyer,** Ind. Sem. Hort. Petrop. 2: 2. 1836. [Type: Cultivated plants grown from seed collected by Douglas, "above the rapids of the Columbia," holotype loc.?]

Stems erect, 1–7 dm high, simple or few branched, spreading hispid below, the stiff hairs with pustular bases, also with shorter and softer, often retrose hairs above, or throughout; leaves linear to linear-oblong or the upper lanceolate, sessile and clasping basally or the lower ones petiolate, 1.5–8 cm long and 1.5 cm wide, often crowded at the base; spikes elongating with age, to 10 cm long, essentially ebracteate; calyx lobes 5, 6–10 mm long in fruit, ciliate and stiffly hairy; corolla yellow to orange-yellow, marked with vermillion in the throat, 6–9 mm long, the throat marked with red, obstructed (nearly closed) by hairy fornices, the limb (3–6?) 5–10 mm wide; stamens inserted below the middle of the tube; style ca 2 mm long, the stigma capitate; nutlets 2.5–3 mm long, ovoid, greenish to dark brown, wrinkled and warty dorsally, the dorsal keel poorly or not at all developed, the scar broadly lanceolate, subbasal (2n =30; n = 15, Ray & Chisaki 1957; 543).

Alaska (Attu Island) and B.C. to w. Montana, south to California (west side of the Sierra Nevada) and Arizona.

The presence of hairy fornices at the opening to the throat of the corolla tube is characteristic of this species. Suksdorf (l.c.; 101) in discussion of the calyx states "3 Lappen mit einander verbunden," although others have described the five calyx lobes as distinct.

Amsinckia menziesii **(Lehmann) Nelson & Macbride**, Bot. Gaz. 61: 36. 1916. [*Echium menziesii* Lehmann Ind. Sem. Hort. Petrop. 2: 29. 1836; Type: N. W. coast of America, Menzies, s.n., isotype BM] Menzies fiddleneck

Stems strictly erect, 1.5–8 dm tall, simple or branched, spreading-hairy with stiff, pustular-based hairs, and often more or less cinereus with fine appressed hairs; leaves linear or lanceolate to oblong,, hirsute on both sides with ascending or appressed hairs, 1.5–10 cm long, 0.5–2.5 cm wide, stiffly hairy, sessile and clasping basally or the lower ones petiolate; inflorescence of 1 or few, strict, erect or ascending racemes, ebracteate; calyx-lobes 5, distinct, 7–13 mm long, linear or linear-lanceolate, stiffly hairy; corolla light yellow, 4–8 mm long, the limb 2–3 mm wide, the throat open, lacking appendages, glabrous; the tube included or only slightly exserted beyond the calyx lobes; stamens inserted above the middle of the tube; nutlets (2) 2.5–3 mm long, broadly ovoid, wrinkled and warty dorsally, the dorsal keel often prominent, at least in the upper portion; scar lanceolate, subbasal (n = 8, 13, 17; Ray & Chisaki 1957;549).

Mixed desert shrub, pinyon-juniper, sagebrush, mountain brush, and aspen-fir communities at 1400 to 2850 m in Beaver, Box Elder, Cache, Davis, Millard, Morgan, Rich, Salt Lake, Tooele, Utah, Wasatch, Washington, and Weber cos.; Alaska and Yukon, s. to Calif. and Nev., east to the Great Plains; also in S. America and in the Old World.

Typically the leaves are linear and have appressed hairs.

Nelson & Macbride (1916; 36) note that the basionym had not then been included within the genus *Amsinckia*, and made the proper nomenclatural combination. Suksdorf (1: 113. 1931) indicates that he did not know this species.

Ganders (Jepson Manual; 368) who includes *intermedia* in an illegitimate combination within *menziesii* notes "100 + named, mostly indistinct variants; self-pollinated; different variants may grow together and remain distinct but intergrade over their ranges."

***Amsinckia retrorsa* Suksdorf,** Deutsche Bot. Monatsschr. 18: 134. 1900. [Type: Washington, Klickitat Co., at Bingen, W. N. Suksdorf 994, holotype?, isotype US, UC, GH. *A. hispidissima* Suksdorf, Deutsche Bot. Monatsschr. 18: 133. 1900: Type: Oregon, Hood River, Suksdorf 2316, holotype?, isotype GH]

Stems erect, simple or few-branched, 1–6 dm tall, spreading-hispid, and also evidently puberulent or strigose throughout with shorter and softer, more or less retrorse hairs; leaves hispid-hirsute with hairs mostly ascending, linear to narrowly oblong, sometimes longer, to 12 cm long, seldom over 1 cm wide, the basal ones often crowded and somewhat larger than the others; spikes elongating with age, often 5–12 cm long (need this measurement); calyx lobes 5, about equal, 5–12 mm long at maturity; corolla orange or orange-yellow, 5–8 mm long (not much exserted from the calyx), the limb mostly 1.5–3 mm wide; nutlets 2–3.5 mm long, ovoid, blackish, muricate-tuberculate and somewhat rugose, with a dorsal ridge, and whitish ventral flange, the scar subbasal, ovate-lanceolate (adapted from Pac NW Fl). n = 8, 13, 17

B.C. to n. Idaho, south to Utah and California

Although placed in synonymy of *A. menziesii* by Ray and Chisaki (l.c.), Cronquist (Intermount. Fl. 1984; 276) distinguishes *A. retrorsa* as a distinct species, differing from both *menziesii* and *intermedia* on the basis of the spreading-hispid hairs of the upper stem being subtended by puberulent or strigose shorter and softer hairs, these more or less retrorse. *A. retrorsa* is also regarded at specific rank in Hitchcock et al. (1959; 181). It is, however, included (in synonymy) within *menziesii* var. *menziesii* in both Munz (1959; 589) and Jepson Fl. Calif (1993; 368).

Suksdorf (l.c.;33) notes that "Diese Nummer werde als A. intermedia F. M.(?) Abgegebeb," in reference to his number 2007, the type of *A. arvensis* Suksdorf.

Cronquist (l.c.; 277) cites *A. eatonii* Suksdorf, Werenda 1: 64. 1931; Type: Utah, Eaton 251, July 1869, holotype CAS, as possibly belonging with *A. retrorsa* also. However, *A. retrorsa* is included in synonymy of *A. menziesii* in A Utah Flora (2010).

Cited as synonyms of *A. retrorsa* in Hitchcock et al. (1959; 181) are Suksdorfian segregate species, whose types are from either Idaho or Oregon. See Appendix list of synonyms.

***Amsinckia spectabilis* Fischer & C. A. Meyer**, Index Seminum (St. Petersburg) 2: 26. 1836; Type: California, Bodega Bay, ["Portia Bodega (aus Albert Pragers Sammlung)," kurzgriffelig, type? CAS ["Ex herbario horti Petropolatam"; de Probe von Meyer selbst bestimmt; langgriffelig," type? UC. fide Suksdorf 1931;97] [*Lithospermum. lycopsoides* Lehmann**,** Stirp. Pug. 2: 28. 1830, not *A. lycopsoides* Lehmann (q.v.); *A. lycopsoides* var. *bracteosa* A. Gray, Syn. Fl. 2(1): 198. 1978, nom. et stat nov. pro *Lithospermum lycopsoides* Lehmann; *A. scouleri* I. M. Johnson, J. Arnold Arbor. 16: 202. 1935, nom. nov. pro *Lithospermum lycopsoides* Lehmann]

Stems erect or decumbent to prostrate, simple or branched, to 4 dm tall, sparsely to moderately hispid with spreading bristles; leaves at least somewhat succulent, often erose-denticulate, lanceolate to lance-linear, lance-elliptic, or lance-oblong, or the lower ones oblanceolate, to ca 5 cm long and 12 mm wide, sessile except the narrowly oblanceolate basal ones; spikes elongating with age, often 5–12 cm long; calyx lobes 5, 4–8 mm long, two of them generally connate below the middle; corolla orange, 4–8 mm long, the limb 2.5–5 mm wide; nutlets 2–2.5 mm long, ovoid, blackish, somewhat rugose or tuberculate, the scar submedian, narrow (adapted from Pac NW Fl et Munz).

Pacific Coast from northern Baja California northward to Washington and Vancouver Island, B.C., and to Skagway and vicinity, Alaska (Welsh & Moore, 8744, 31 Jul 1968; 8794, 1 Aug 1968, collections previously labeled as *A. menziesii*)

Ray and Chisaki (l.c.) indicate that the homostylous "*A. spectabilis* have spread far north and south on maritime sand dunes along the Pacific coast, but have not penetrated inland more than a few miles anywhere," and note further that "all the names cited under *A. spectabilis* and its varieties by Hoover are considered synonyms of species in the present interpretation." Thus, the following cited entities at varietal rank have been variously interpreted.

var. *sancti-nicolai* (Eastwood) Johnston ex Munz, Man. S. Calif Bot. [Munz] 423. 1935. [*A. sancti-nicolai* Eastwood, Proc. Calif Acad. III. 1(3): 109. 1898; *A. intermedia* var. *sancti-nicolai* (Eastwood) Jepson, Man. Fl. Pl. Calif [Jepson] [5]: 844. 1925 (as var. *nicolai*); Type: California, sands, San Nicolas Island, Blanche Trask 58, April 1901, holotype CAS]

Spike bracteate throughout! Endemic to San Nicolas, San Miguel, and San Clemente Islands, California. In synonymy of var. *spectabilis* in Jepson Manual (1993; 368), but maintained as distinct by Abrams (l.c.;606).

var. *microcarpa* (Greene) Jepson & Hoover, Fl. Calif. (Jepson) 3: 326. 1943. [*A. microcarpa* Greene, Erythea 2: 191. 1894; Type: California, sans loc., Coulter 497, holotype GH?] Calyx lobes all distinct to base or only slightly connate; corolla 13–16 mm oblong. Nutlets 1–2 mm long, muriculate, but not usually with a dorsal ridge.

Near the coast, San Luis Obispo and nw Santa Barbara Cos., California.

Stems erect, calyx lobes distinct; nutlets 1–1.5 mm; flowers heterostylous..... var. *microcarpa*

Stems generally decumbent, calyx with 2 or 3 lobes connate below the middle; nutlets 1.5–2 mm; flowers heterostylous or not .....var. *spectabilis*

(Key modified from Jepson Manual)

***Amsinckia tessellata* A. Gray**, Proc. Amer. Acad. Arts 10: 54. 1874. [Type: California, "Contra Costa Mountains near Mt. Diablo, W. H. Brewer" 1119, in 1862, holotype GH, isotypes UC!, US] Rough fiddleneck

Stems stout, branched throughout or sometimes simple below, 3–6 dm high, hispid with spreading bristles; leaves

linear-lanceolate, 2–7 cm long, rather thinly hispid, the hairs pustulate at base, sessile except the narrowly oblanceolate basal ones; spikes elongating with age, often 5–12 cm long; calyx lobes (2) 3 or 4, when 4 with 1 broader and notched or 2-lobed at apex, when 3 a little broader and notched at apex, hispid and on the margins densely white-hirsute, 8–13 mm long; corolla orange, 5–10 mm long, the limb 2.5–5 mm wide; nutlets 3–3.5 mm long, ovoid, the back low and usually with a median line, densely tessellate or papillate, and often transversely rugose, the scar lanceolate, basal or nearly so.

Creosote bush, Joshua tree, mixed warm desert shrub, sagebrush, oak, and pinyon-juniper communities at 750 to 1900 m in Box Elder, Davis, Iron, Juab, Millard, Salt Lake, Sevier, Summit, Tooele, Utah, Washington, and Weber cos.; Wash. to Ida., Ariz., and Baja Calif.; 61 (xiii).

Rough fiddleneck is one of three species cited by Hitchcock et al. (l.c.;177) along with *lycopsoides* and *spectabilis* as "sharply limited and technically marked." Still, from within its variability, Suksdorf and other authors were able to distinguish a great number of apparently inconsequential segregate "species."

Pac. N.W. Flora (1959; 181) cites three Suksdorf segregate species as synonyms of *A. tessellata*, all from Oregon and Washington.

***Amsinckia vernicosa* Hooker & Arnot,** Bot. Beechey Voy. 370. 1839 (collections of Lay & Collie). [Type: California, Douglas s.n., sans date, GH (type?)]

Stems erect, branched throughout or sometimes simple below, 2–6 dm high, sparsely setose and pustulate, or nearly glabrous; leaves lanceolate to lance-ovate, 4–8 cm long, somewhat clasping at the base, somewhat glaucous and fleshy, abundantly pustulate above; spikes elongating with age, 3–12 cm long; calyx lobes (2) 3 or 4, setose dorsally, the margins pale-ciliate, 9–18 mm long; corolla orange, 10–12 mm long, golden yellow, the limb 3–14 mm wide, the tube 20-veined below attachment of stamens; nutlets 4–6 mm long, ovoid, gray, smooth and shining, the scar lineate "attached at the lower part of the sharp inner angle by a narrow scar, all three faces plane or nearly so" (Gray 1978;197). (2n=14).

Corolla 3–8 mm broad, yellow; nutlets lanceolate in outline with an entire groove..... var. *vernicosa*

Corolla 8–14 mm broad, orange; nutlets ovate-lanceolate, with forked groove ..... var. *furcata*

var. *furcata* (Suksdorf) Hoover in Jepson, Fl. Calif. (Jepson) 3: 326. 1943. [basionyn: *A. furcata* Suksdorf, Werenda 1: 113. 1931; Type: California, Cuyama white hills near the boundary between Santa Barbara and San Luis Obispo counties, Alice Eastwood s.n., 1 May 1896, holotype UC] Forked fiddleneck

Corolla 12–22 mm long, the limb 8–14 mm wide, orange; nutlet scar forked at base (n=14). Plant rare; Inner South Coast Ranges, in San Benito, Fresno, Kings, and San Louis Obispo cos., Calif. (Ray and Chisaki 1957;530, treated as a species); California endemic.

var. *vernicosa* Corolla 8–12 mm long, the limb 3–8 mm wide, yellow; nutlet scar unforked (2n=14). Greenhorn Mts, Kern Co., west side of San Joaquin Valley, and adjacent Coast Ranges from Alameda to San Luis Obispo County and desert ranges in San Bernardino and Inyo cos. (Ray & Chisaki l.c.) to Monterey and Fresno counties, California, endemic.

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Brand A. 1929. Decas specierum novarum nona. *Repertorium Specierum Novarum Regni Vegetabilis* 26: 168-172. *Amsinckia toussaintii* Brand, based on plants grown in Germany from seeds from Chile!; *A. santae barbarae* Brand, p. 168, "Mediocriter hispida." Caulis erectus strictus simplex vel parse ramosus 30-70 cm altus. Folia linearia 35-70 mm longa, 7-10 mm lata apice acuta. Cincinni terminaler densi et multiflori basi tantum bracteatei; sepals

lineari subulata, 4-5 mm long, fulvo-setosa; corolla infundibuliformis 8-10 mm longa 4-7 mm lata; stamina fauci affixa; stylis calyci fructifero 7-8 mm longo subaequilongus vel eo bervior. Nuculae brunneae vel fuscae late muricatae, fauci interiore muricateae, utrinque carinatae; cicatrix minima. "Calafornien: Santa Barbara, Elwood (Eastwood no 8. Herb Berlin)," the first cited! Also cited are: "Santa Clara County: Stanford University (Baker no 391, vermischt mit *A. Campestris).* – Blüt im Marz und April."

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Evaluation of the Stability of Benzofuran Ketones in Rayless Goldenrod (*Isocoma pluriflora*) and White Snakeroot (*Ageratina altissima*) Under Different Storage Conditions

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Abstract

White snakeroot (*Ageratina altissima*) and rayless goldenrod (*Isocoma pluriflora*) cause "trembles" and "milk sickness" in livestock and humans, respectively. The toxin in white snakeroot and rayless goldenrod was identified in 1927 and 1930, respectively, as tremetol. It was reported that the toxin in white snakeroot disappears as it is dried and that completely dried plants were incapable of producing "trembles" or "milk sickness." However, it has been suggested that the toxins in rayless goldenrod were not degraded by drying and both fresh and dry plants are toxic. Later, tremetol isolated from white snakeroot and rayless goldenrod was determined to be a complex mixture containing various benzofuran ketones including tremetone **1**, dehydrotremetone **2**, and hydroxytremetone in white snakeroot and tremetone **1**, dehydrotremetone **2**, and 3-oxyangeloyltremetone **3** in rayless goldenrod. In this report, the stability of the benzofuran ketones in white snakeroot and rayless goldenrod was studied by measuring the concentrations of the benzofuran ketones in ground and in intact dried leaves stored at different temperatures over an approximately 6-year time period.

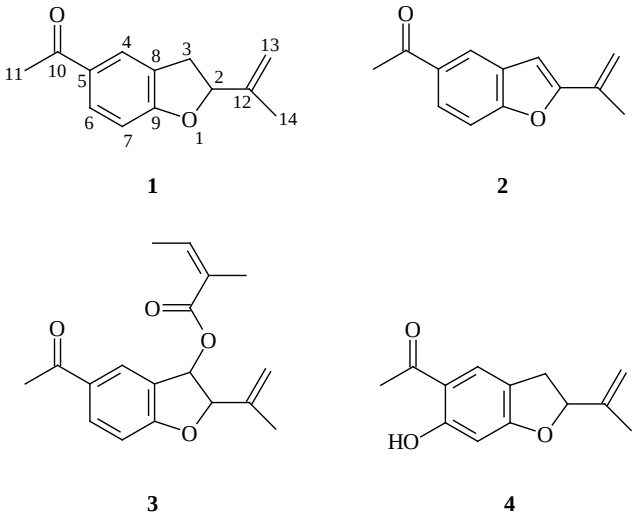
Keywords: *Ageratina altissima*, dehydrotremetone, *Eupatorium rugosum, Isocoma pluriflora*, rayless goldenrod, tremetone, white snakeroot

Introduction

White snakeroot (*Ageratina altissima* (L.) R.M. King & H. Rob. var. *altissima*) (family, Asteraceae) and rayless goldenrod (*Isocoma pluriflora* (Torr. & A. Gray) Greene) (family, Asteraceae) cause "trembles" and "milk sickness" in livestock and humans. "Milk sickness" caused many deaths among Midwestern settlers during the 1800s, forcing entire settlements to be abandoned (Couch 1927, Kingsbury 1964, Burrows and Tyrl 2013). In 1917, white snakeroot was shown to cause "trembles" and "milk sickness" (Moseley 1917). Poisoning in livestock is first manifest as depression, reluctance to eat, and inactivity followed by muscle tremors of the nose, flanks, and legs especially after exercise. The poisoned animal will often display rapid breathing, elevated heart rate, a stiff gait, and altered posture as affected animals are reluctant to move and stand hunched up with a flexed back. In the early 1900s, a disease with nearly identical clinical signs as trembles broke out in livestock in the southwestern United States, and it was quickly established that the southwestern "milk sickness" was due to ingestion of rayless goldenrod (Marsh 1926; Couch 1927, 1930).

Couch identified the toxins in white snakeroot and rayless goldenrod as tremetol (Couch 1927, 1930). Later, Couch reported that tremetol rapidly disappeared when white snakeroot was dried and that completely dried plants were incapable of producing "trembles" or "milk sickness" (Couch 1926,1927, 1928, 1930). Couch suggested that toxin degradation was plant specific as he concurrently

reported that the rayless goldenrod toxins were not destroyed by drying, and therefore both fresh and dry plants were toxic (Couch 1926, 1927, 1930). Later, tremetol isolated from white snakeroot and rayless goldenrod was determined to be a complex mixture containing various benzofuran ketones including tremetone **1**, dehydrotremetone **2**, and hydroxytremetone in white snakeroot (Bonner et al. 1961, Bonner and DeGraw 1962) and tremetone **1**, dehydrotremetone **2**, and 3-oxyangeloyltremetone **3**, in rayless goldenrod (figure 1) (Zalkow et al. 1962, 1979). Several studies have implicated the benzofuran ketones as the toxic principles although there is no conclusive large animal data to support this suggestion (Bonner et al. 1961; Zalkow et al. 1962, 1979; Bowen et al. 1963; Beier et al. 1987, 1993).



1 **2**

3 **4**

Figure 1. Chemical structures of tremetone **1**, dehydrotremetone **2**, 3-oxyangeloyltremetone **3**, and 6-hydroxytremetone **4**.

In a recent study, the effect of different plant drying methods on the concentrations of the benzofuran ketones in white snakeroot and rayless goldenrod was evaluated (Lee et al. 2012). It was concluded that the benzofuran ketones were most stable when they were freeze dried and air dried but showed some instability when they were oven dried in a period of 7 days between collection, drying, and analysis. More recently, we surveyed several *Isocoma* spp. for their benzofuran ketone content using field and herbarium specimens (Lee et al. 2015). In general, a lower frequency of detection of several benzofuran ketones was observed in herbarium specimens compared with field collections. In particular, it was observed that dehydrotremetone **2** was present in the majority of the field collections but was conspicuously absent in many of the herbaria specimens. This observation was further investigated by sampling and analyzing a herbarium specimen that was over 5 years old and by comparing it with plant material from the same location that we had analyzed over 5 years previously. This comparison suggested that the benzofuran ketones tremetone **1**, dehydrotremetone **2**, and 3-oxyangeloyltremetone **3** had degraded over the 5-plus years and further suggested that dehydrotremetone **2** degraded more rapidly than the other benzofuran ketones. Based on these observations, the objective of this study was to determine the stability of the benzofuran ketones in white snakeroot and rayless goldenrod in ground and in intact plant material at different temperatures over an approximately 6-year time period.

Experimental

Plant Material

Rayless goldenrod was collected May 6, 2008, near Pecos, TX, 31º23.969´ N / 103º29.969´ W, accession #3056 at the ARS Poisonous Plant Research Laboratory (PPRL) Herbarium, Logan, UT, and accession #250012 at the Utah State University Intermountain Herbarium, Logan, UT. White snakeroot was collected September 5, 2008, at Hart Woods, IL, 40º13.732’ N / 88º21.346’ W, accession #3401 at the PPRL Herbarium. Plants were identified by PPRL personnel.

Plant Processing

The plants from the rayless goldenrod collection were dried at ambient temperature, and the leaves stripped off and collected in a 4 L plastic utility pan and thoroughly mixed by stirring the leaves by hand and then separated into six subsamples: room temperature-unground, refrigerator-unground, freezer-unground, room temperature-ground, refrigerator-ground, and freezer-ground. The subsamples (approximately 2 g) were placed in re-sealable zipper plastic bags. Room temperature samples were stored in the laboratory at 21 °C, refrigerated samples were stored at 4 °C, and freezer samples were stored at –20 °C. All subsamples were stored in the dark. Plants from the white snakeroot collection were treated in the same manner as the rayless goldenrod plants and separated into the same six subsamples described above.

Rayless goldenrod and white snakeroot samples designated ground were ground to pass through a 1

mm screen using a Cyclotec 1093 sample mill (Tecator, Hoganas, Sweden) on February 17, 2009, and February 18, 2009, respectively. Rayless goldenrod and white snakeroot samples designated unground were ground the day of extraction, 1 day prior to the date of HPLC analysis.

Extraction

Dry, ground leaf material was weighed (100 mg) into a screw-top glass test tube (16 mL). The leaf material was extracted (16 h) by mechanical rotation with hexane:ethyl acetate (8 mL, 70:30 v:v). The samples were centrifuged (5 min at 15,000 x *g*) and supernatant transferred (1 mL) into autosampler vials for HPLC analysis. Rayless goldenrod and white snakeroot samples from different storage conditions were run in sextuplicate (n = 6) on days 0 and 2119 and run in singlet on days 7, 21, 35, 56, 85, 140, 252, and 525.

HPLC

Analytical scale reversed phase HPLC was performed on a Shimadzu LC-20AT equipped with an autosampler and PDA detector from the same vendor and a 100 mm x 2 mm i.d., 5 µm, Betasil C_{18} column (Thermo Hypersil-Keystone, Bellefonte, PA). Samples (10 µL) in the extraction solution were injected on to the column and eluted with a 20 mM ammonium acetate-acetonitrile mobile phase at a flow rate of 0.4 mL/min. The mobile phase program was 20 mM ammonium acetate-acetonitrile (65:35, v:v) for 4 min followed by a linear gradient to a composition of 65% acetonitrile at 20 min. At 21 min, the composition was increased to 100% acetonitrile for 5 min. Detection of analytes in the eluent was performed at λ 280 nm. The compound concentrations in leaves were quantitated against tremetone **1**, dehydrotremetone **2**, 3-oxyangeloyltremetone **3**, and 6-hydroxytremetone **4** standards. Purity of standards was > 95% as determined by HPLC-PDA and NMR. Six-point calibration curves of the standards were prepared by serial dilution using previously isolated **1**, **2**, **3**, and **4** (Lee et al. 2009) in hexane:ethyl acetate (70:30, v:v) over the range of 3.13 µg/mL – 100 µg/mL.

Data Analysis

A one-way ANOVA was performed using Sigma Plot 12.5 where chemical amounts in the different storage conditions were compared. A post-hoc test of significance using a Bonferroni correction was performed. A p-value of <0.05 was considered to be statistically significant.

Results and Discussion

The qualitative and quantitative benzofuran ketone profile in rayless goldenrod and white snakeroot at day 0 was similar to that previously reported by Lee et al. (2009) (figure 2). Three benzofuran ketones, tremetone **1**, dehydrotremetone **2**, and 3-oxyangeloyltremetone **3**, were detected in rayless goldenrod (table 1), while three benzofuran ketones, tremetone **1**, dehydrotremetone **2**, and 6-hydroxytremetone **4**, were detected in white snakeroot under all storage conditions and time points (table 1). Temperature, state of the plant material (ground or unground), and storage time all influenced the stability of each benzofuran ketone in rayless goldenrod and white snakeroot. Each benzofuran ketone responded to these factors independently although some general trends were observed. First, each respective compound decreased more in both species in the samples stored at 21 °C compared to the sample stored at −20 °C in ground and unground samples, while samples stored at 4 °C were generally intermediate. For example, in rayless goldenrod that was not ground, dehydrotremetone **2** amounts at day 2119 were 1.3 ±0.1, 0.49 ±0.08, and 0.10 ±0.01 µg/mg in the respective samples stored at −20, 4, and 21 °C (table 1). Second, each respective compound decreased to a greater extent in the ground sample of each species compared to the unground sample of each species stored at the same temperature. For example, in white snakeroot stored at 4 °C, tremetone **1** amounts at day 2119 were 0.71 ±0.02 µg/mg in the ground sample and 2.7 ±0.2 µg/mg in the unground sample (table 1). Third, degradation of each compound was not linear over the experiment. In general, most of the degradation of the benzofuran ketones occurred within the first 252 days (figures 3-8). For example, concentrations of dehydrotremetone **2** in rayless goldenrod were stored at 21 °C were 1.2 ± 0.1, 0.34, and 0.10 ± 0.01 µg/mg in the unground sample at day 0 (initial), day 252, and day 2119 (figure 4).

Dehydrotremetone **2** is the most rapidly degraded of the four benzofuran ketones in rayless goldenrod and white snakeroot in this study (figures 2, 4, 7). Dehydrotremetone **2** degraded in both plants under all storage conditions ($P<0.05$) with one exception, unground rayless goldenrod stored at −20 °C. For example, over 83% of the dehydrotremetone **2** in rayless goldenrod is degraded over the total study time of 2,119 days in unground plant material at 21 °C (figure 4). Of the 83% of the degraded dehydrotremetone **2**, 69% of the total loss occurs in

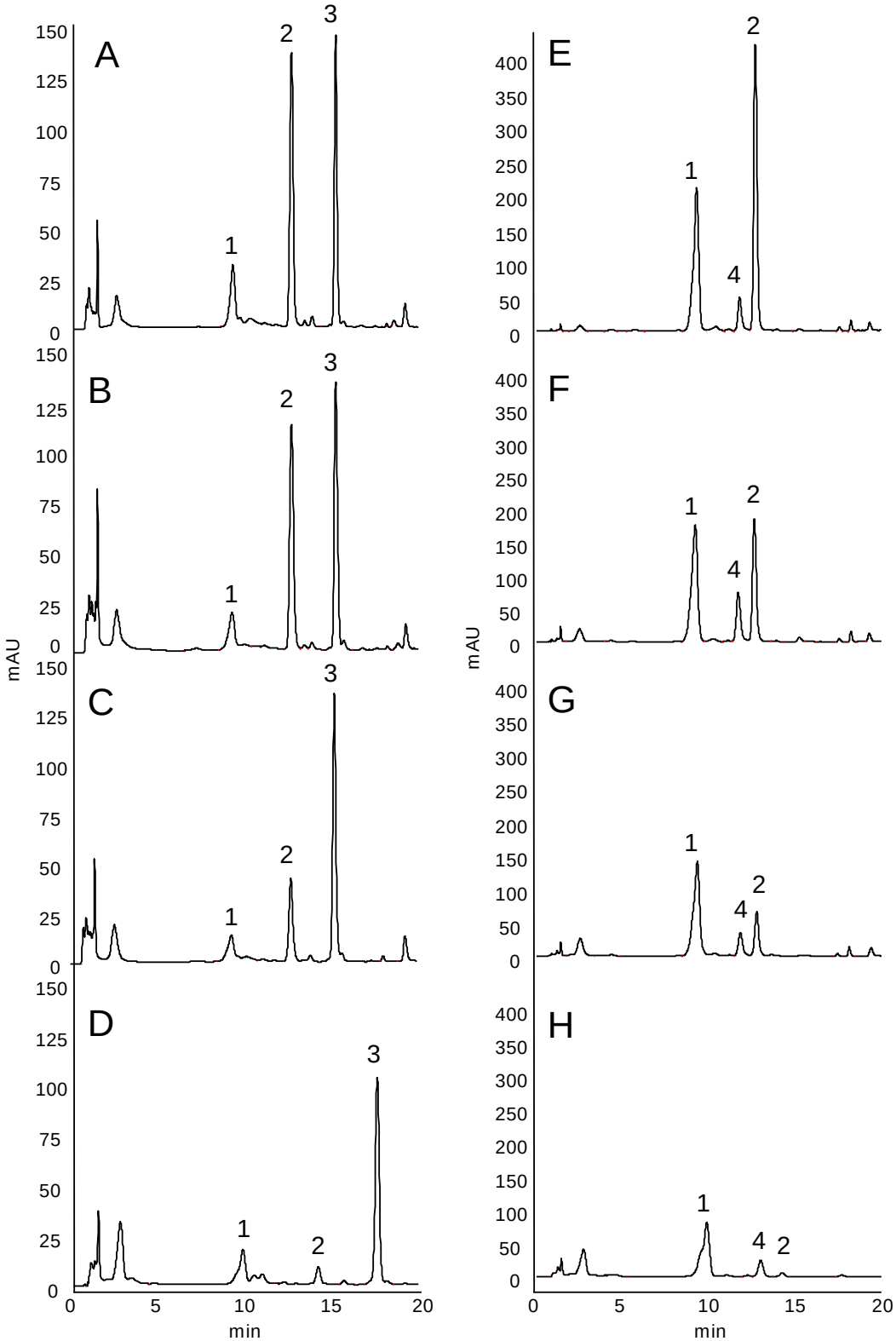


Figure 2. HPLC chromatograms of unground rayless goldenrod stored at 21 °C at (A) day 0, (B) day 85, (C) day 252, and (D) day 2119. HPLC chromatograms of unground white snakeroot stored at 21 °C at (E) day 0, (F) day 85, (G) day 252, and (H) day 2119. Annotated peaks correspond to tremetone **1**, dehydrotremetone **2**, 3-oxyangeloyltremetone **3**, and 6-hydroxytremetone **4**. Peak retention times for the day 2119 chromatogram are different due to a different Betasil C_{18} column used in the analysis.

Table 1. Variation in mean concentrations (±SD) of benzofuran ketones in rayless goldenrod and white snakeroot over 2,119 days under different storage temperatures[1]

Storage Conditions	Compound Concentrations ± SD (n=6) µg/mg of dry weight		
Rayless Goldenrod	Tremetone **1**	Dehydrotremetone **2**	3-Oxyangeloyltremetone **3**
Initial	0.57 ± 0.03[b]	1.2 ± 0.1[a]	5.3 ± 0.4[b]
Unground -20 °C	0.73 ± 0.04[a]	1.3 ± 0.1[a]	6.5 ± 0.3[a]
Unground 4 °C	0.48 ± 0.08[c,d]	0.49 ± 0.08[b]	5 ± 1[b]
Unground 21 °C	0.40 ± 0.03[d,e]	0.10 ± 0.01[d]	5.3 ± 0.2[b]
Ground -20 °C	0.55 ± 0.02[b,c]	0.50 ± 0.01[b]	4.8 ± 0.1[b]
Ground 4 °C	0.41 ± 0.02[d]	0.27 ± 0.01[c]	3.66 ± 0.04[c]
Ground 21 °C	0.33 ± 0.02[e]	0.031 ± 0.002[d]	1.7 ± 0.1[d]
White Snakeroot	Tremetone **1**	Dehydrotremetone **2**	6-Hydroxytremetone **4**
Initial	5.3 ± 0.9[a]	3.5 ± 0.6[a]	1.2 ± 0.3[b]
Unground -20 °C	4.8 ± 0.2[a]	2.4 ± 0.1[b]	1.5 ± 0.1[a]
Unground 4 °C	2.7 ± 0.2[b]	0.88 ± 0.05[c]	0.68 ± 0.09[c]
Unground 21 °C	2.1 ± 0.2[b,c]	0.07 ± 0.01[e]	0.45 ± 0.07[c]
Ground -20 °C	1.75 ± 0.03[c]	0.66 ± 0.01[c,d]	0.50 ± 0.01[c]
Ground 4 °C	0.71 ± 0.02[d]	0.30 ± 0.01[d,e]	0.171 ± 0.004[d]
Ground 21 °C	0.30 ± 0.01[d]	0.0145 ± 0.0004[e]	0.040 ± 0.001[d]

[1]Different letters within a column represent significance between drying methods at $P < 0.05$.

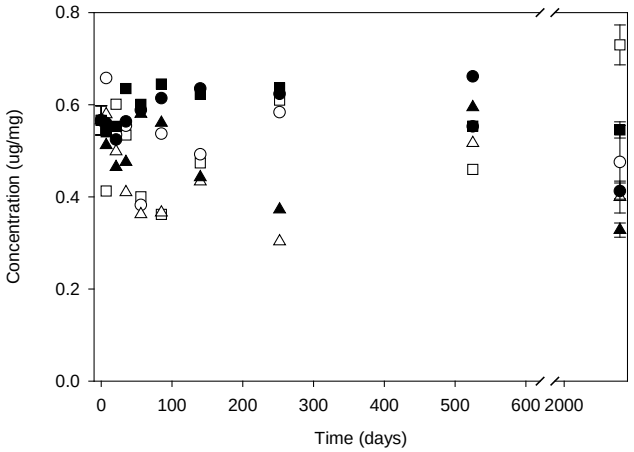


Figure 3. Variation in the concentration of tremetone **1** in rayless goldenrod over time under different storage conditions: unground −20 °C (□); unground 4 °C (○); unground 21 °C (Δ); ground −20 °C (■); ground 4 °C (●); and ground 21 °C (▲).

the first 252 days. Similar relative amounts of dehydrotremetone **2** degradation were observed in white snakeroot. Tremetone **1** and 6-hydroxytremetone **4** showed similar trends of degradation under similar conditions but to a lesser extent than dehydrotremetone **2**. The most stable of the benzofuran ketones was 3-oxyangeloyl-tremetone **3**. No significant degradation of 3-oxyangeloyltremetone **3** was detected in any of the samples with the exception of ground samples stored at 4 and 21 °C.

In summary, the four benzofuran ketones were most stable when stored as unground material at −20 °C while they were most unstable when stored as ground material at 21 °C regardless of the plant matrix. All benzofuran ketones showed no or minimal degradation when stored at −20 °C in both rayless goldenrod and white snakeroot with the exception of dehydrotremetone **2** in white snakeroot. These results are consistent with our observations of tremetone **1**, dehydrotremetone **2**, and 3-oxyangeloyltremetone **3** in herbarium specimens compared with field collections in different *Isocoma* spp. (Lee et al. 2015). The occurrence of 3-oxyangeloyltremetone **3** was the most consistently observed benzofuran ketone between herbarium specimens and recent field collections while dehydrotremetone **2** was rarely observed in the older herbarium specimens. In a previous study, minimal degradation of the benzofuran ketones under

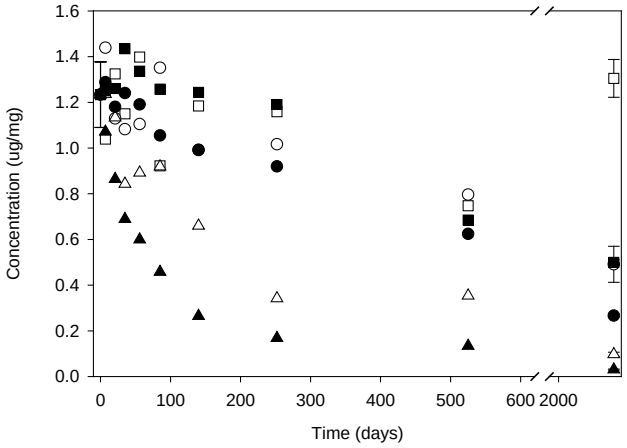


Figure 4. Variation in the concentration of dehydrotremetone **2** in rayless goldenrod over time under different storage conditions: unground −20 °C (□); unground 4 °C (○); unground 21 °C (Δ); ground −20 °C (■); ground 4 °C (●); and ground 21 °C (▲).

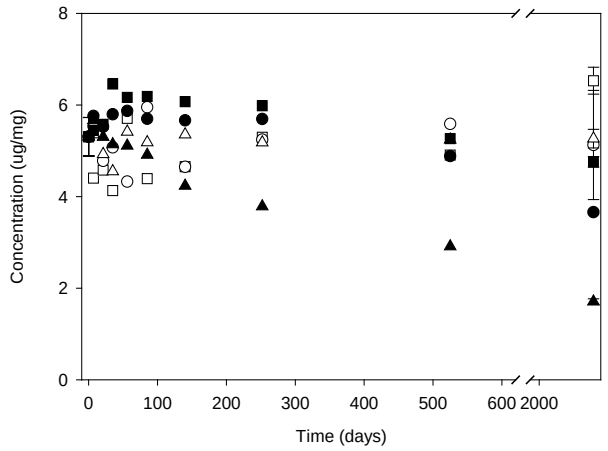


Figure 5. Variation in the concentration of 3-oxyangeloyltremetone **3** in rayless goldenrod over time under different storage conditions: unground −20 °C (□); unground 4 °C (○); unground 21 °C (Δ); ground −20 °C (■); ground 4 °C (●); and ground 21 °C (▲).

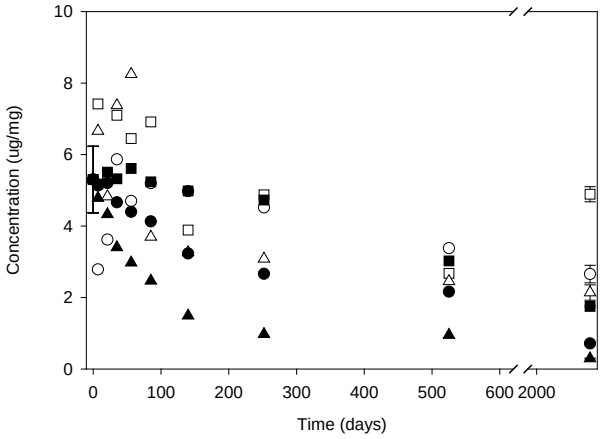


Figure 6. Variation in the concentration of tremetone **1** in white snakeroot over time under different storage conditions: unground −20 °C (□); unground 4 °C (○); unground 21 °C (Δ); ground −20 °C (■); ground 4 °C (●); and ground 21 °C (▲).

different drying conditions was observed in rayless goldenrod and white snakeroot; however, the length of time between drying, grinding, and analysis was 7 days (Lee et al. 2012). As a result, we were unable to observe the degradation of the benzofuran ketones observed here over a longer period of time.

The instability of the benzofuran ketones is due to factors within the plant as these compounds are highly stable when purified (Lee et al. 2012). Since the benzofuran ketones have been reported to contribute to the toxicity of these plants, in considering these results, plants should be stored intact before and ground just prior to animal dosing studies. Lastly, these results may contribute to the reported sporadic toxicity of these plants made by early investigators.

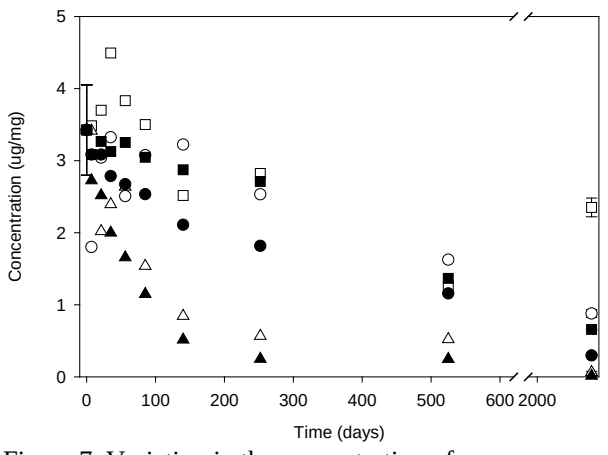


Figure 7. Variation in the concentration of dehydrotremetone **2** in white snakeroot over time under different storage conditions: unground −20 °C (□); unground 4 °C (○); unground 21 °C (Δ); ground −20 °C (■); ground 4 °C (●); and ground 21 °C (▲).

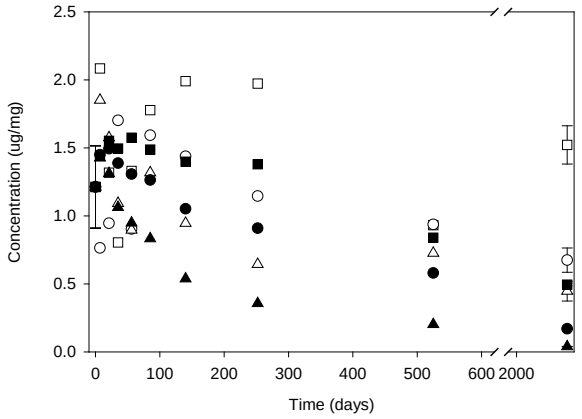


Figure 8. Variation in the concentration of 6-hydroxytremetone **4** in white snakeroot over time under different storage conditions: unground −20 °C (□); unground 4 °C (○); unground 21 °C (Δ); ground −20 °C (■); ground 4 °C (●); and ground 21 °C (▲).

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An Evaluation of the Toxicity of White Snakeroot (*Ageratina altissima*) and Rayless Goldenrod (*Isocoma pluriflora*) in a Lactating Mouse Model

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Abstract

Rayless goldenrod (*Isocoma pluriflora*) and white snakeroot (*Ageratina altissima*) have been implicated in the poisoning of various livestock species including cattle, sheep, goats, and horses, as well as humans. It has also been observed that nursing young can be poisoned when their mothers consume these plants, indicating that the toxins are excreted in the milk. Both plants contain similar benzofuran ketones, which have been suggested to be the toxins. However, recent research suggests that another compound (or other compounds) may be responsible. Consequently, additional research needs to be done to definitively identify and characterize the toxic component(s) of these plants. The objective of this study was to determine if a lactating mouse model can serve as a good small-animal model to study the toxicity of white snakeroot and rayless goldenrod, with the end goal of identifying the toxin(s) in these plants. White snakeroot and rayless goldenrod were dosed orally, via the chow, to lactating dams from post-natal days (PND) 1-21. The pups were evaluated for locomotor activity as well as motor coordination and function on PND 14, 21, and 28. There was no indication of muscle weakness or tremors in any of the dams or their pups. The results from this study suggest that neither white snakeroot nor rayless goldenrod is toxic to lactating mice or their pups. Consequently, mice do not appear to be a viable small-animal model to study the toxicity of these plants.

Abbreviations: BFK, benzofuran ketones; PND, post-natal day; RGR, rayless goldenrod; WSR, white snakeroot.

Keywords: *Ageratina altissima*, benzofuran ketones, BFK, *Isocoma pluriflora*, mice, rayless goldenrod, white snakeroot

Introduction

Rayless goldenrod (RGR, *Isocoma pluriflora*) is a toxic range plant found in the southwestern United States and northern Mexico commonly growing in dry, open sites especially in alkaline areas (Burrows and Tyrl 2001, 2013). White snakeroot (WSR, *Ageratina altissima*) is found throughout the eastern half of North America, usually in low, moist, partially shaded sites such as wooded areas (Burrows and Tyrl 2001, 2013). Both plants have been implicated in the poisoning of various livestock species including cattle, sheep, goats, and horses, as well as humans (Beier and Norman 1990). The consumption of milk from cows grazing WSR caused many deaths among Midwestern settlers during the 1800s, at times forcing entire settlements to be abandoned (Couch 1927). In 1917, WSR was shown to be responsible for “trembles” and “milk sickness” (Couch 1927, Moseley 1941). Cases of

milk sickness in humans who have consumed milk from cows grazing WSR have been reported as recently as 1963 (Hartmann et al. 1963). In the early 1900s, a disease with nearly identical clinical signs broke out in the southwestern United States, and it was quickly established that the southwestern "milk sickness" was due to ingestion of RGR (Marsh 1926). Both WSR and RGR have been shown experimentally to poison nursing livestock (Wolf et al. 1918, Marsh 1926).

Both WSR and RGR contain a substance historically referred to as "tremetol," which causes "trembles" and "milk sickness" in livestock and humans, respectively (Couch 1927). Tremetol has been shown to be a complex mixture of sterols and derivatives of methyl ketone benzofuran that includes, but is not limited to, tremetone **1**, dehydrotremetone **2**, 3-oxyangeloyl-tremetone **3**, and 6-hydroxytremetone **4** (figure 1) (Bonner et al. 1961, Bonner and DeGraw 1962, Lee et al. 2009). The toxicity of RGR and WSR is due to myoskeletal and myocardial degeneration and necrosis (Stegelmeier et al. 2010, Davis et al. 2013a,b). Consequently, the most characteristic clinical sign of poisoned animals is muscle tremors, which is accentuated by exercise. Toxicity occurs after consumption of 0.5-1.5% of the animal's body weight during a 1-3 week period (Kingsbury 1964; Davis et al. 2013a,b, 2015).

Although the benzofuran ketones (BFK) have traditionally been thought to be the toxic components of WSR and RGR, recent research by our group suggests that another compound (or other compounds) may be at least partially responsible. In a recent study using yearling goats, the BFK were extracted from WSR, adsorbed onto both alfalfa and the extracted residue at the same concentrations as in the WSR plant material, and dosed to goats. The goats dosed with ground WSR plant were poisoned; however, the goats dosed with BFK adsorbed onto alfalfa and BFK adsorbed on to the previously extracted WSR plant residue were not poisoned, suggesting that another compound(s) responsible for the poisoning was lost or modified in the extraction process (Davis et al. 2015). Consequently, additional research needs to be done to definitively identify and characterize the toxic component(s) of these plants. The fact that nursing young can be poisoned when their mothers eat these plants, or when humans drink milk from cows that have consumed toxic WSR, indicates that the toxic components are passed through the milk. Therefore, the objective of this study was to determine if a lactating mouse model can serve as a good small-animal model to study the toxicity of WSR and RGR, with the end goal of identifying the toxin(s) in these plants.

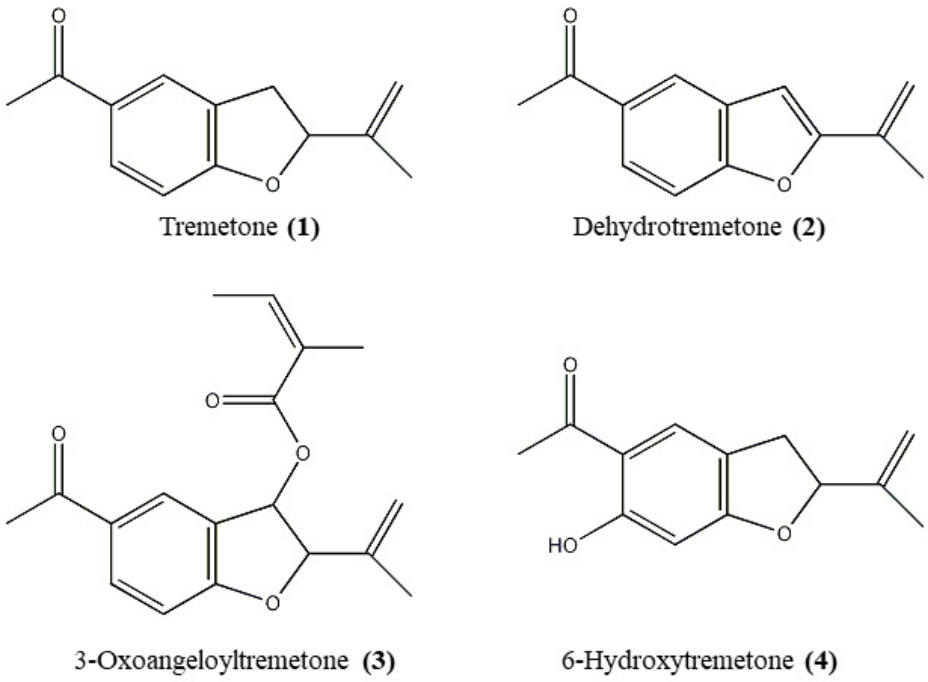

Figure 1. Structure of the benzofuran ketones identified in white snakeroot and rayless goldenrod.

Materials and Methods

Plant Material

White snakeroot (WSR, *Ageratina altissima*) was collected in the early to mid-flowering stage during September 2010 near Champaign, IL (N40 05.54 W87 49.68, at an elevation of 181 m). Rayless goldenrod (RGR, *Isocoma pluriflora*) was collected in the pre-flowering stage during May 2010 near Pecos, TX (N31 23.56 W103 29.58, at an elevation of 788 m). Both plants were air dried and stored intact in a protected enclosure. Immediately prior to preparing rodent chow, the plants were ground to pass through a 2 mm screen using a Wiley Mill. Alfalfa (*Medicago sativa*) was harvested in June 2014 near Logan, UT (N41 54.82 W111 48.57, at an elevation of 1425 m). The alfalfa was harvested using normal agriculture practices of swathing, drying, and baling the hay. The dried alfalfa hay was ground to pass a 2 mm screen using a Gehl Mix-All model 55 (Gehl Company, West Bend, WI, USA).

Animals

All procedures were conducted under veterinary supervision and were approved by the Utah State University Institutional Animal Care and Use Committee. Male and female Swiss Webster mice (8 weeks old) were purchased from Simonsen Laboratories Inc., Gilroy, CA. Mice were acclimated for 3 to 4 d with free access to a commercially pelleted rodent chow (Harlan Teklad rodent diet (w) 8604) and tap water before beginning experiments. Mice were housed under controlled temperature (20-22 °C) in a 12:12 h light:dark cycle. Mice were hand mated (3 females and 1 male per cage) for 12 h each night. Once the females were visibly pregnant, they were housed individually for the remainder of the study. The mice were dosed as outlined below beginning on post-natal day (PND) 1 through PND 21. The mice were weaned on PND 21, with each litter of pups housed as a group for the remainder of the study.

Diets were prepared using the same commercially pelleted rodent chow (Harlan Teklad rodent diet (w) 8604), which was ground and mixed with 10% corn starch and either ground WSR or RGR in hot water to obtain chow containing 75%, 50%, 25% WSR and 40%, 20%, 10% RGR. Pellets, approximately 1 x 3 cm in size, were formed and air dried overnight at room temperature (~ 21 °C). The chow for the control group was prepared in the same manner but with 90% commercial rodent chow and 10% corn starch. Additionally, a plant control pellet was prepared using 50% alfalfa, 10% corn starch, and 40% commercial rodent chow. Chow consumption, maternal body weight, and the number of pups in each litter were measured every 2 days beginning on PND 1 and through PND 21.

HPLC Analysis

Using an HPLC method developed for the quantitation of BFK in WSR and RGR (Lee et al 2009), the concentrations of **1**-**4** were determined in dry, ground plant material and pelleted rodent chow containing WSR or RGR. Ground plant or pelleted material was weighed (100 mg) into a screw-top glass test tube (16 mL). The plant and pellet material was extracted (16 h) by mechanical rotation with 8 mL hexane:ethyl acetate (70:30 v:v) at ambient temperature. HPLC was performed using a Shimadzu LC-20AT (Shimadzu Co., Kyoto, Japan) equipped with an autosampler and PDA detector from the same vendor and a 100 mm x 2 mm i.d., 5 µm, Betasil C_{18} column (Thermo Hypersil-Keystone, Bellefonte, PA). Samples (10 µL) in hexane:ethyl acetate (70:30 v:v) were injected onto the column and eluted with a 20 mM ammonium acetate-acetonitrile mobile phase at a flow rate of 0.4 mL/min. The mobile phase program was 20 mM ammonium acetate-acetonitrile, 65:35 v:v for 4 min followed by a linear gradient to a composition of 65% acetonitrile at 20 min. At 21 min, the composition was increased to 100% acetonitrile for 5 min. The eluant was monitored at λ=280 nm. Under these conditions, tremetone **1**, dehydrotremetone **2**, 3-oxyangeloyltremetone **3**, and 6-hydroxytremetone **4** eluted at 9.8, 14.2, 17.4, and 12.9 min, respectively. The concentrations of **1**-**4** in plant material and pellet material were quantified against a seven-point calibration curve using previously isolated tremetone **1**, dehydrotremetone **2**, 3-oxyangeloyltremetone **3**, and 6-hydroxytremetone **4** (Lee et al. 2009). The standard solutions were prepared by serial dilution with hexane:ethyl acetate (70:30) over the range of 0.39 µg/mL-200.0 µg/mL. The calibration curves had R^2 < 0.9997.

Rotarod

An accelerating rotarod apparatus (IITC Life Science Inc.) was used to assess motor function (Jones and Roberts 1968, Crawley 1999). The apparatus had 5 lanes, and rod diameter was 3.2 cm. Mice were tested on PND 21 and 28 in a balanced

order across treatments. Animals were briefly trained to walk on the rotarod immediately prior to the PND 21 test. The apparatus was set to accelerating mode from 3 to 40 rpm in 300 sec, reaching a maximum at 180 sec. The mice were positioned in their respective lanes while the rods were not moving. Once all the mice were positioned, the rods began accelerating. The latency to fall was recorded automatically for each lane. The individual test of any animal was terminated if it exhibited a passive rotation whereby it was hanging by its forelimbs from the rod during a complete rotation. Each animal was given two consecutive trials on the rotarod at each time period, with an inter-trial interval of 30 sec after the last mouse fell.

Open Field Analysis

The open field apparatus is a common measure of exploratory and anxiety behavior in mice. In the context of this study, our interest centered on evaluating motor impairment or changes in locomotor activity (Kręz˙el et al. 1998). Any-maze® software (San Diego Instruments) was used in conjunction with four open fields (50 x 50 cm, 38 cm wall height) to test four pups simultaneously. Pups were tested in the open field for 10 minutes on PND 14, 21, and 28. An overhead camera and the software automatically tracked the movement of each animal in the open field.

Statistical Analysis

Statistical comparisons were performed using ANOVA with a Bonferroni posthoc test of significance between individual groups using SigmaPlot for Windows (version 12.5, SPSS Inc., Richmond, CA). Correlations were determined with a Spearman Rank Order Correlation analysis using SigmaPlot for Windows (version 12.5, SPSS Inc., Richmond, CA). Differences were considered statistically significant at $P < 0.05$. Four pups per litter were evaluated on each apparatus, unless there were fewer than four pups in the litter. In those instances, all of the remaining pups (1-3) were evaluated. The values for the litter were averaged, and the litter was considered the experimental unit for statistical analyses.

Results and Discussion

Both RGR and WSR have been known to be poisonous to livestock and humans for many years. They both cause very similar clinical signs of poisoning, including muscle weakness and tremors. Additionally, they both contain BFK. Even though it has been suggested that the BFK are the toxic components of these plants, this has not been demonstrated in a mammalian model. To this end, a number of small-animal models have been previously evaluated, including several rodent species (Bowen et al. 1963, Beier and Norman 1990). All research to date suggests that adult rodents, with guinea pigs possibly an exception, are not a good model to study the toxicity of these plants (Bowen et al. 1963, Davis unpublished data and personal communications). It is, however, quite common for neonatal animals to be more sensitive to toxins than mature, adult animals (Whitney et al. 1995, Zimmerman 1999, Piñeiro-Carrero and Piñeiro 2004). Therefore, the objective of this study was to determine if a lactating mouse model can be used to study the toxicity of RGR and WSR.

The BFK profiles of the RGR and WSR used for this study are shown in figure 2. The RGR contained tremetone **1**, dehydrotremetone **2**, and 3-oxyangeloyltremetone **3**, with **3** accounting for the majority of the BFK. The WSR contained tremetone, dehydrotremetone, and 6-hydroxytremetone, with similar amounts of tremetone and 6-hydroxytremetone. The plants were dosed orally to lactating dams via their chow. Commercial rodent chow was mixed with the plants to make chow that consisted of 75%, 50%, and 25% WSR and 40%, 20%, and 10% RGR. Higher percentages of WSR were used because preliminary experiments indicated that mice will more readily eat rodent chow containing WSR than RGR (data not shown). The concentration of BFK in the various batches of chow is shown in table 1.

Mice were fed treated lab chow from PND 1 through PND 21. During this period, chow consumption, maternal weight, and the number of pups in each litter were recorded. There was significantly less chow consumed in all treatment groups compared to controls, except for the alfalfa control chow (figure 3). Similarly, there was significantly less chow consumed in all other groups compared to alfalfa control chow, except for the chow containing 20% RGR. In general, there was a trend for less chow consumption with increased percentage of plant in the chow for both WSR and RGR. The decreased chow consumption translated to a daily weight loss in the 75% WSR, 50% WSR, and 40% RGR groups (figure 4). Finally, all of the mice dosed with WSR chow received a much higher dose of BFK than the RGR-dosed mice (figure 5).

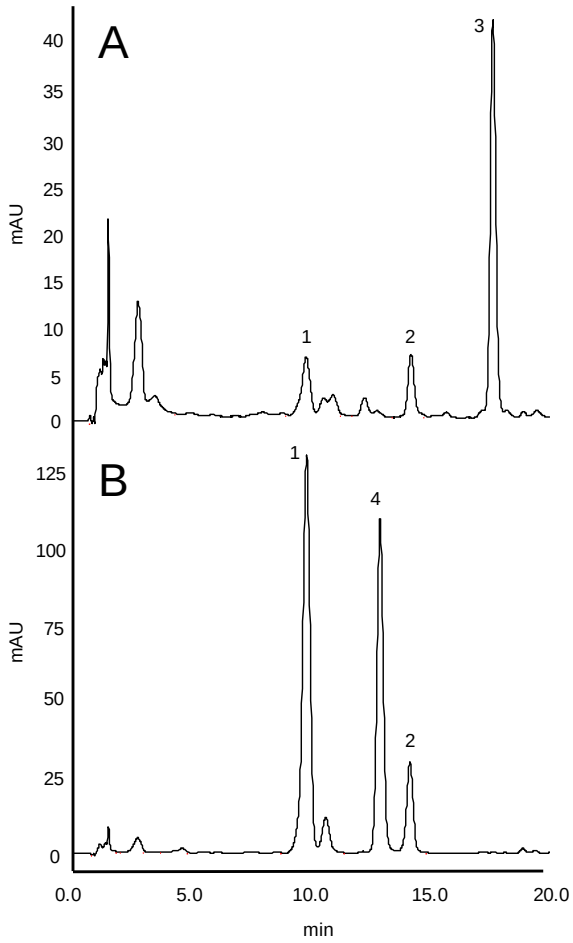

Figure 2. HPLC chromatograms of the benzofuran ketones in rayless goldenrod (A) and white snakeroot (B). mAU, milli-absorbance units.

The number of pups in each litter was counted every other day from PND 1 to PND 21 to determine if the treatments affected the survival rate of the pups. There was a significant decrease in the survival of pups in all treatment groups compared to the controls, including the alfalfa control group (figure 6A). Most notable were the 75% WSR, 50% WSR, and 40% RGR groups, which lost all of their pups within 4 to 7 days on treatment. There was also a high mortality rate observed in the 25% WSR group, with 95% of the pups dying. Interestingly, the pups in the alfalfa control group also saw a significant mortality with 75% of the pups dying. Only the 75% WSR, 50% WSR, and 40% RGR groups had a significantly higher mortality rate than the alfalfa control group. The mortality rate in the dams was also noted (figure 6B). None of the dams in the control, alfalfa control, 40% RGR, 20% RGR, or 10% RGR groups died, whereas there was 100%, 83%, and 29% mortality in the dams from the 75% WSR, 50% WSR, and 25% WSR groups, respectively. It is interesting to note that while all of the pups in the 40% RGR group died, none of the dams died. These data indicate that the WSR is either more toxic to the dams than RGR or that RGR is more nutritious to mice than WSR.

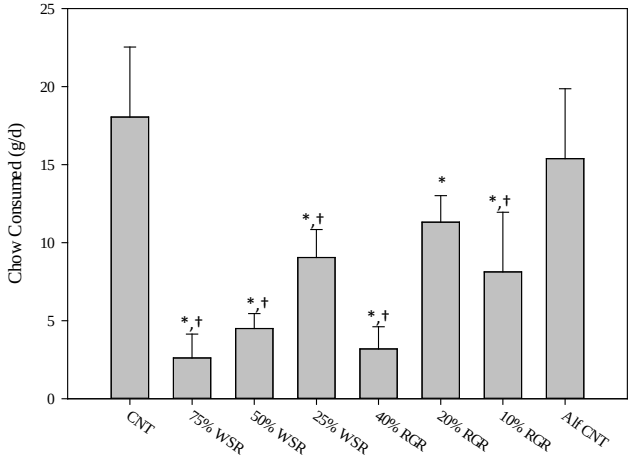

Figure 3. Daily chow consumption. Data represent the mean ± SD for the daily chow consumption from post-natal day (PND) 1-21 (n = 6-7 dams per group). *indicates statistical significance compared to control group. †indicates statistical significance compared to alfalfa control group. WSR is white snakeroot, and RGR is rayless goldenrod.

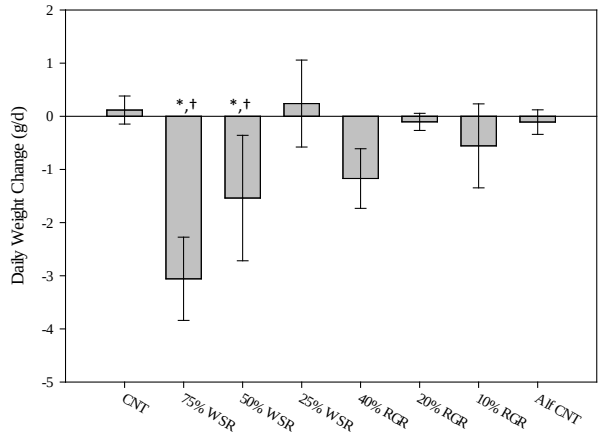

Figure 4. Maternal daily weight change. Data represent the mean ± SD for the maternal daily weight change from post-natal day (PND) 1-21; n = 6-7 dams per group. *indicates statistical significance compared to control group. †indicates statistical significance compared to alfalfa control group. WSR is white snakeroot, and RGR is rayless goldenrod.

In order to assess any muscle weakness that may have occurred in the pups during the treatment period, we analyzed the pups using rotarod and open field analyses. Of important note, no overt visual signs of muscle weakness including muscle tremors were observed in any of the pups from any of the

Table 1. The concentration of benzofuran ketones in rodent chow

Plant	% Plant[a]	mg benzofuran ketone[b] / g chow				
		Tremetone	Dehydro tremetone	6-Hydroxy tremetone	3-Oxyangeloyl tremetone	Total[c]
White snakeroot	75	1.35	0.14	1.29	n.d.[d]	2.78
	50	0.76	0.10	0.74	n.d.	1.59
	25	0.43	0.06	0.40	n.d.	0.89
Rayless goldenrod	40	0.03	0.03	n.d.	0.17	0.22
	20	0.02	0.02	n.d.	0.13	0.16
	10	0.01	0.01	n.d.	0.07	0.09

[a]Percentage of plant material in the rodent chow.
[b]The benzofuran ketones analyzed included tremetone, dehydrotremetone, 6-hydroxytremetone, and 3-oxyangeloyltremetone.
[c]The total is a sum of the three individual benzofuran ketones detected in each plant.
[d]n.d. = not detected.

treatment groups. The pups from 75% WSR, 50% WSR, and 40% RGR groups were not evaluated on the rotarod nor open field due to the fact that they all died within 7 days of the beginning of the treatment. We evaluated the pups from the control, alfalfa control, 25% WSR, 20% RGR, and 10% RGR groups in the open field on PND 14, 21, and 28. The primary aim of the open field evaluation was to determine if any of the treatments affected the pups' ability to move normally in terms of distance traveled, average speed of movement, maximum speed of movement, and total time mobile. There was no difference in these four measurements at PND 14 or 28 in the pups from any of the groups (figure 7). There was however, a significant decrease in the distance traveled, average speed, and total time mobile in the pups from the 25% WSR group on PND 21. The pups were evaluated for muscle coordination and function using a rotarod. The pups were not evaluated on PND 14 as they were not able to adequately walk on the rotating rod at this age. Consequently, evaluations were only made on PND 21 and 28. Again, the pups from the 25% WSR group were not able to walk on the rotating rod for as long as the controls at both periods of evaluation (figure 8). There were no differences in the ability of the pups from any of the other treatment groups to walk on the rotarod compared to the control or alfalfa control groups.

There were only three pups in the 25% WSR group from six litters that survived (95% mortality rate). All three mice were lethargic and did not move around normally (figures 8 and 9). Although the pups from the 25% WSR group did not perform as well as the pups from the other treatment groups, there was no indication that this was due to muscle weakness, and there were no signs of muscle tremors in these pups.

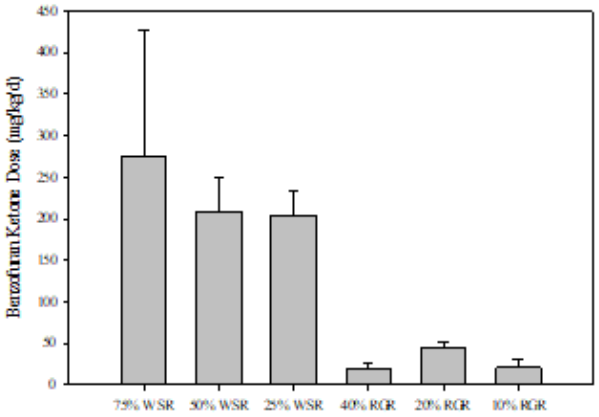

Figure 5. Daily dose of benzofuran ketones. Data represent the mean ± SD for the daily dose of all benzofuran ketones (mg/kg/d), n = 6-7 dams per group. WSR is white snakeroot, and RGR is rayless goldenrod.

Although we did not see any evidence of muscle damage or weakness/trembling in the mice, we did observe that the chow containing high amounts of the plants had a negative effect on the survival of the pups (figure 6). We found a significant positive correlation between the survival rate of the pups and both the amount of chow the dams consumed ($r = 0.854$, $p = 0.002$) as well as the weight change in the dams ($r = 0.810$, $p = 0.01$) (figure 9). This suggests that the dams that ate more chow were able to maintain their body weight better and thus were able to provide the proper nourishment for their pups to survive. There was no correlation between pup survival and the total BFKs dose ($r = -0.479$, $p =$

0.207), indicating that the BFKs did not contribute to the pup mortality. Therefore we suggest that the adverse effects observed such as increased mortality were due to poor nutrition in the lactating dams fed chow containing large portions of either plant and not toxicity of the BFKs.

In conclusion, the data from this study suggest that neither WSR nor RGR is toxic to lactating mice or their pups. Given the fact that these plants are known to be toxic to nursing livestock, mice do not appear to be a viable small-animal model to study the toxicity of RGR or WSR. The results of this study suggest that all adverse effects observed such as increased mortality were due to poor nutrition in the lactating dams fed chow containing large portions of either plant. Future work will continue to try and identify a suitable small-animal model that can be used to identify and characterize the toxic compounds in WSR and RGR.

Acknowledgments

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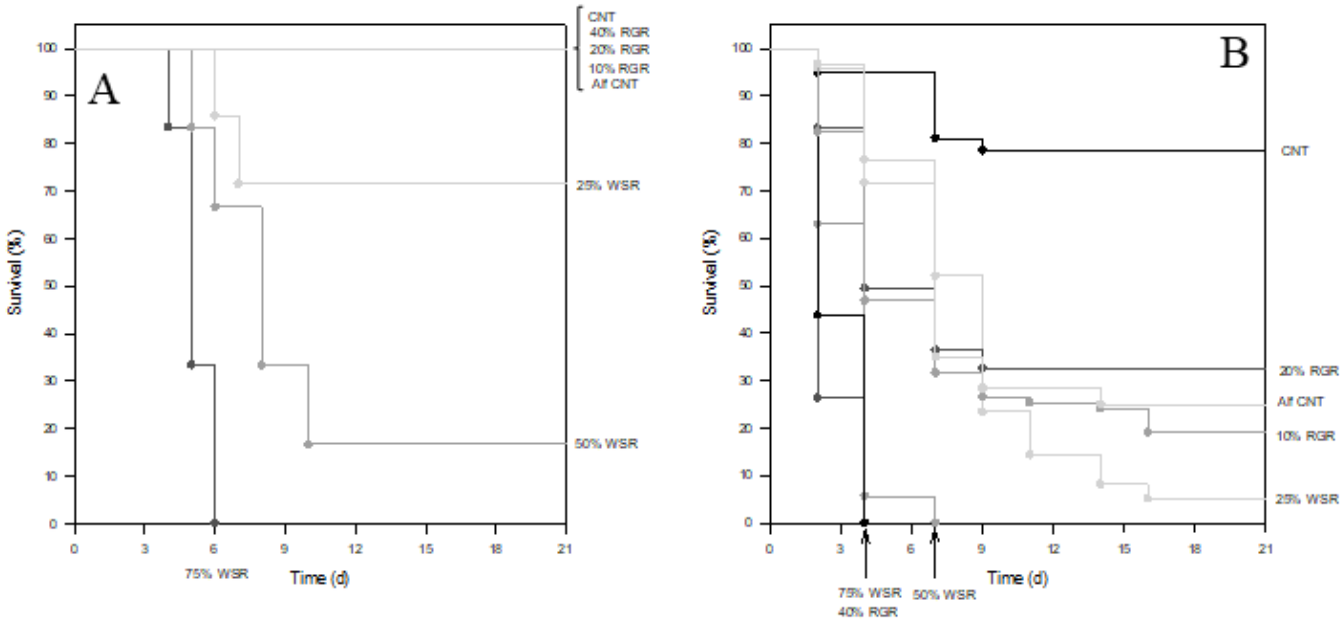

Figure 6. Effect of rayless goldenrod (RGR) and white snakeroot (WSR) treatment on pup and dam survival. Data represent the percent of surviving animals over time. At the beginning of the experiment, there were 60-98 pups per group and 6-7 dams per group.

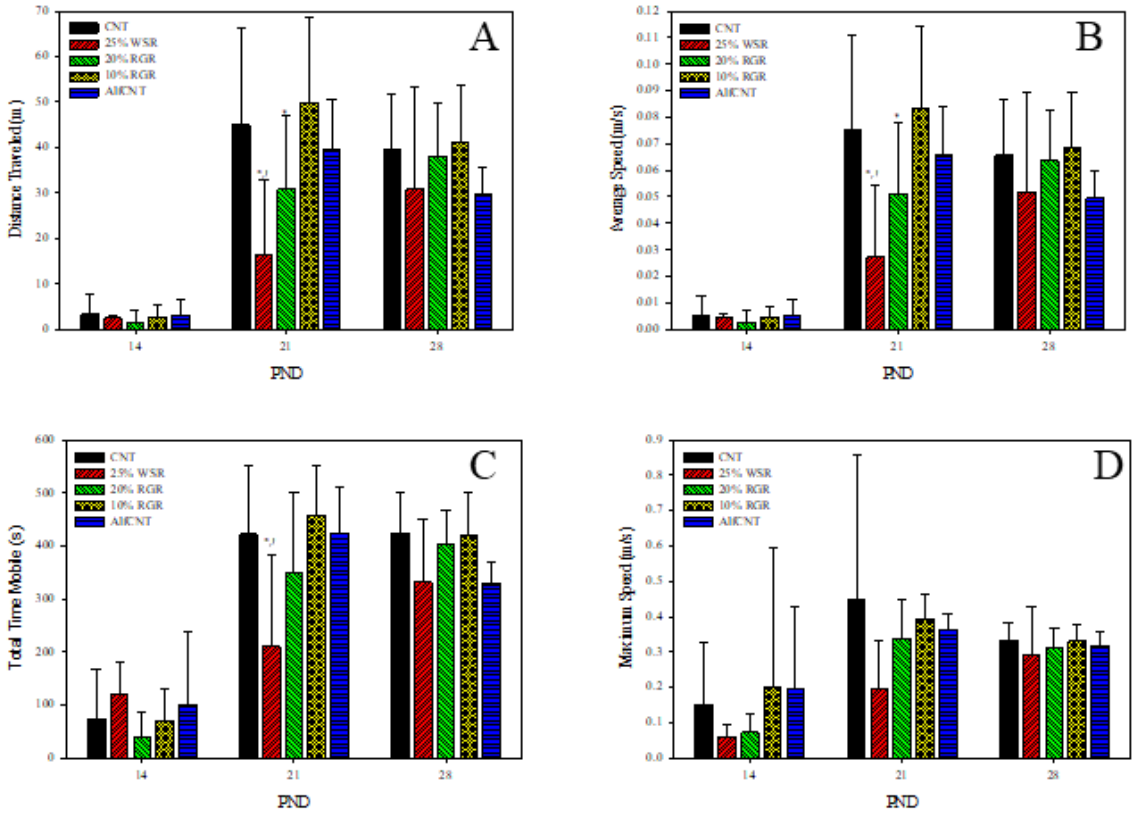

Figure 7. Effect of rayless goldenrod (RGR) and white snakeroot (WSR) treatment on locomotor activity. Locomotor activity of the pups was assessed via open field analysis of distance traveled (A), average speed of movement (B), total time mobile (C), and maximum speed of movement (D) on post-natal day (PND) 14, 21, and 28. Data represent the mean ± SD, n of 6-7 dams per group. *indicates statistical significance compared to control group. †indicates statistical significance compared to alfalfa control group.

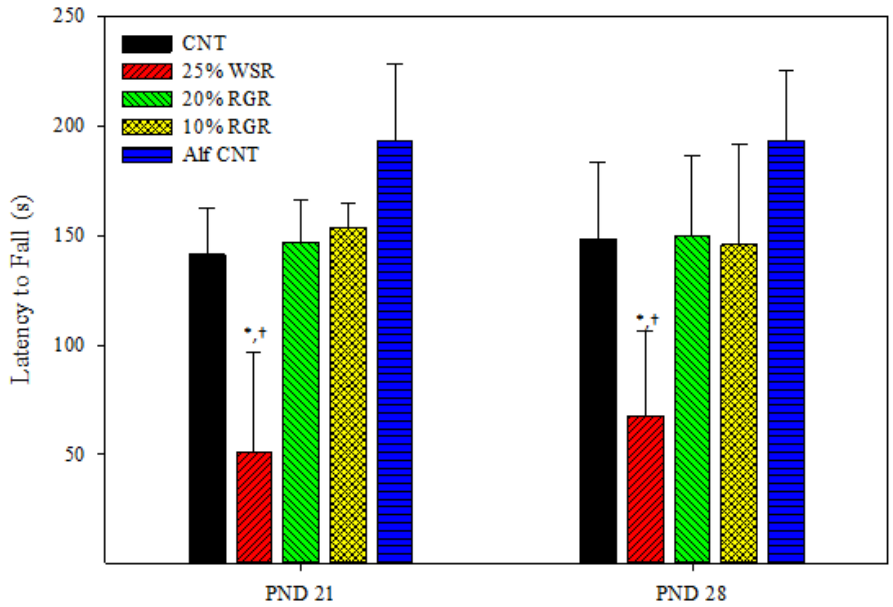

Figure 8. Effect of rayless goldenrod (RGR) and white snakeroot (WSR) treatment on motor coordination and function on post-natal day (PND) 21 and 28. Motor coordination and function were assessed by evaluating the ability of pups to walk on a rotating rod. Data represent the mean ± SD for the latency to fall from the rod, n of 6-7 dams per group. *indicates statistical significance compared to control group. †indicates statistical significance compared to alfalfa control group.

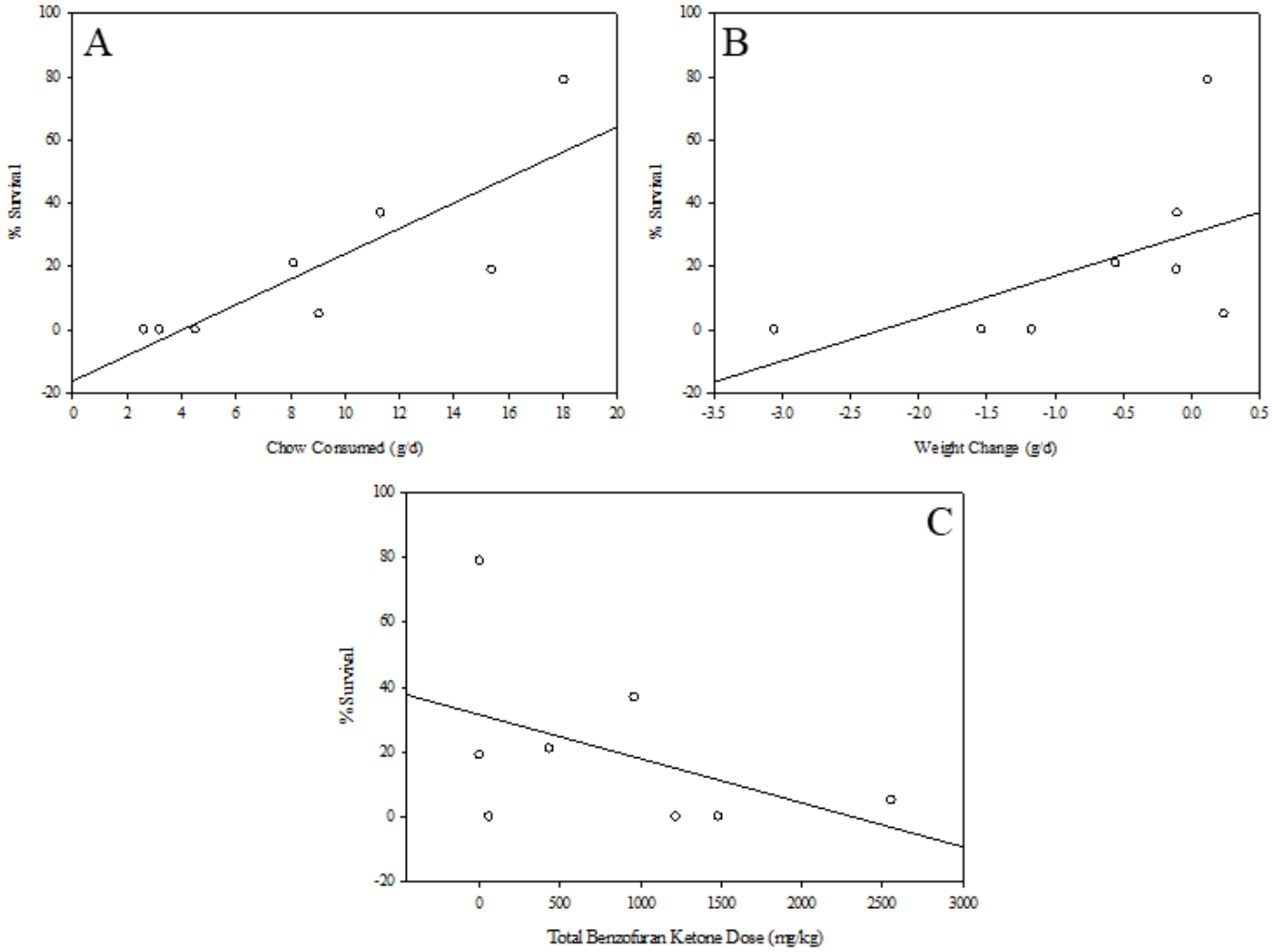

Figure 9. Correlation of pup survival with maternal chow consumption, maternal weight change, and total benzofuran ketone dose.

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ELISA-Based Correlation of *Rathayibacter toxicus* Antigen With Corynetoxins in Pasture and Hay

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Abstract

Enzyme-linked immunosorbent assays (ELISAs) for a *Rathayibacter toxicus* antigen and for corynetoxins were developed in the 1990s and in 2000-2002, respectively, to assist in the management and control of annual ryegrass toxicity (ARGT). The relationship between the results of these two ELISAs has not previously been established. This relationship was examined in an inter-laboratory study and in samples collected from the field in Western Australia (WA). The inter-laboratory study compared the results obtained by three laboratories, each having performed both ELISAs on the same samples. There was good agreement between laboratories in results for both analytes. The correlations between pairs of laboratories for the *R. toxicus* ELISA were r = 0.86, 0.85, and 0.92; and for the corynetoxins ELISA the correlations between pairs of laboratories were r = 0.98, 0.97, and 0.98. The results of the corynetoxins ELISA were also compared with those of the *R. toxicus* antigen ELISA in infected annual ryegrass and hay samples collected from paddocks, windrows, and hay bales on four different properties in WA at various times throughout spring and summer. The inter-laboratory study and the analysis of field samples described in this paper show that *R. toxicus* antigen and corynetoxins are present simultaneously in the analyzed samples and that there is an overall positive correlation: r = 0.70 ($p<0.001$, n = 100) and 0.80 ($p<0.001$, n = 389) in the two studies respectively between *R. toxicus* antigen and corynetoxins concentrations in extracts of ryegrass and hay samples.

Keywords: ARGT, annual ryegrass toxicity, immunoassay

Introduction

Annual ryegrass toxicity (ARGT) occurs when livestock graze on pastures or ingest hay containing annual ryegrass (*Lolium rigidum*) infected by the bacterium *Rathayibacter toxicus* (Sasaki et al. 1998). This bacterium produces corynetoxins (Payne et al. 1983) that, if ingested in sufficient quantities, will cause neurological signs and subsequent death in livestock (McKay et al. 1993). A parasitic nematode (*Anguina funesta*) carries *R. toxicus* into the growing ryegrass plant. The nematodes induce the formation of galls, instead of seeds, inside the developing seedheads, and multiply within these structures. The gall and seedhead may be colonized by *R. toxicus* when sufficient numbers of the bacterium are present (figure 1). These bacterial galls contain a yellow mass of toxin-producing bacteria (Bird and Stynes 1977, Vogel et al. 1981). In vitro studies have

shown that corynetoxins are mainly produced when bacterial growth is in the stationary phase, although some corynetoxins are detectable at the end of log-phase growth (Payne and Cockrum 1988). In vivo, the quantity of corynetoxins in bacterial galls is implicitly expected to be proportional to the bacterial load and the weight of the galls. Indeed, analysis of Cockrum and Edgar's (1985) data shows a high correlation between gall weight and corynetoxins content (r = 0.91). The production of the corynetoxins is greatest when the plants senesce (Stynes and Bird 1983).

Figure 1. (top) Annual ryegrass (*L. rigidum*) seedheads and (bottom) from left: two dehusked *R. toxicus* bacterial galls, three bacterial galls, two nematode galls, two *L. rigidum* seeds. [Magnification 10x]. Courtesy of Leo den Hollander.

ARGT has been reported in South Australia (SA), Western Australia (WA), and South Africa (McIntosh et al. 1967, Gwynne and Hadlow 1971, Schneider 1981). *R. toxicus* has also been implicated in stock losses because of its presence in galls induced by *Anguina paludicola* (Bertozzi and Davies 2009) in blowngrass, *Lachnagrostis filiformis* (formerly *Agrostis avenacea*, Jacobs 2001) in northern New South Wales, and annual beardgrass, *Polypogon monspeliensis*, in southeastern SA (McKay and Ophel 1993). *R. toxicus* infection in these grasses is similar to that in annual ryegrass, and the same corynetoxins are produced (Edgar et al. 1994). In addition, corynetoxins have been implicated in stock poisoning related to nematode seed galls of *Festuca nigrescens* in the United States and New Zealand (Anderton et al. 2004a). In 1996, ARGT was diagnosed in cattle in Japan after they had eaten hay imported from WA. This incident resulted in the introduction of a voluntary hay testing protocol to minimize contamination of exported hay with *R. toxicus* (Elson 2002a,b). The protocol was made compulsory in 2005.

The hay-testing protocol requires laboratories to provide a sensitive, high-throughput assay for the presence of *R. toxicus*. The assay in use since 1996 has been an enzyme-linked immunosorbent assay (ELISA) for a water-soluble antigen of *R. toxicus* (Masters et al. 2006, 2011). Since this ELISA could only be regarded as providing an indirect measure of the toxicity of samples, the Commonwealth Scientific and Industrial Research Organisation (CSIRO) developed an ELISA that quantitates the amount of corynetoxins in samples (Than et al. 2002). An inter-laboratory study was performed in 2002-2003 to compare the robustness of the two ELISAs by testing the same samples in three different laboratories. Since the bacterial antigen ELISA protocol formed the backbone of the hay-testing protocol, and since the more complex corynetoxins ELISA was a direct measure of the potential for toxicity, the correlation of corynetoxins to the level of bacterial antigen—and hence the validation of the bacterial antigen ELISA—was investigated in the inter-laboratory study and in additional samples collected from the field in WA.

Materials and Methods

Bacterial Galls

R. toxicus bacterial galls were separated from ryegrass samples collected in 1999 from an infected location in the Wongan Hills-Ballidu district of WA. The ryegrass was threshed, and the galls were identified and sorted visually over a light box by two independent operators. One operator did the initial sorting, and a second checked the selected galls and removed any that were not bacterial galls (figure 2).

Corynetoxin Standards

The corynetoxins (figure 3) used as analytical standards and to develop and validate the corynetoxins ELISA were isolated from grain screenings collected during harvest in WA in the

Figure 2. Sorting galls over a light box. Courtesy of Leo den Hollander.

early 1990s. The extraction and purification process afforded the corynetoxins with a purity of >95% (Anderton et al. 2004b). Briefly, the grain screenings were sieved to concentrate the bacterium-colonized galls that were subsequently extracted with 80% methanol in water. After evaporation of the solvent, the crude extract was treated with aqueous sodium hydroxide to hydrolyse co-extracting glycerides (Frahn et al. 1984). Adapting the method of Vogel et al. (1981), acidification of the hydrolysis mixture resulted in a precipitate enriched in corynetoxins that was subsequently partitioned with ethyl acetate and water to produce an insoluble, more-enriched corynetoxin fraction at the ethyl acetate/water interface. Further solvent-solvent partitioning using a phase transfer reagent and final preparative HPLC, based on the analytical HPLC described by Cockrum and Edgar (1985), provided the pure corynetoxins in gram quantities (Anderton et al. 2004b). Similar to the confirmation of corynetoxins in bacterial "galls" from New Zealand and the United States (Anderton et al. 2004a), the identities of the individual corynetoxins were further confirmed by diverting the effluent from the HPLC column to a LCQ ion trap mass spectrometer (Thermo-Finnigan) operated in the positive ion, electrospray ionization mode. Prior to the analysis, the mass spectrometer was tuned as per operating guidelines by infusing a solution of a purified, major corynetoxin (figure 3).

Samples for Inter-Laboratory Study

One hundred oaten hay samples were obtained from hay export companies in WA (Gilmac Pty Ltd) and SA (Balco Australia Pty Ltd and Golden Plains Fodder Australia Pty Ltd). The samples were collected by farm staff on various farms early in 2001 from hay bales made in the year 2000 with a steel hay-bale core sampler attached to the front or rear of a 4-wheel-drive vehicle. The hay-bale core samplers were not commercially available but were custom made based on the design of those used for bale core sampling for nutritional testing. The hay-bale core samplers were cylindrical, 35±10 mm in diameter, and >800 mm long. There was a collection hole at the vehicle end of the sampler, and a plastic collection bag was attached under the hole so that the hay sample was forced into the bag when the core sampler was driven 800 mm into the bale (figure 4). Cores (~50 g) were collected from each end of 10 bales, all into the same collection bag, so that each final composite sample weighed 1000±200 g. Residual hay was cleaned out of the core sampler after collection of each composite sample.

Field Samples

Samples were collected from four farms: farm A (in the Brookton Shire, where ARGT had not been reported and *R. toxicus* had not been detected) and farms B, C, and D (in the Shires of Victoria Plains and Serpentine-Jarrahdale, respectively, where ARGT had occurred and a high prevalence of *R. toxicus* in extracts of pasture and hay samples had been detected by use of the *R. toxicus* antigen ELISA in the previous 2 years). Oaten hay (on farms A and B) or meadow hay (on farms C and D) and standing ryegrass samples were collected. Hay exporters (farms A and B) and dairy farmers (farms C and D) cut 5-10 g ryegrass samples or ryegrass heads from fence lines immediately adjacent to areas to be cut for hay at 10 meter intervals until 200 g in total was collected per fence line. Samples were collected every 14 days from July 2001 to January 2002 (except on farm D: July to end of November 2001) from a total of 21 fence lines on the 4 farms (2 on farm A, 10 on farm B, 4 on farm C, and 5 on farm D; most of the fence lines being in different paddocks). After cutting the pasture for hay, random grab samples were collected from the windrows until 1 kg of composite sample was collected per paddock (twice per week). Once the hay was baled, sampling was to be performed as for the inter-laboratory study except that at each collection time, the sample on each paddock or farm was a 200g core taken from one end of a single bale. This was done from mid-November 2001 to the middle of February 2002 on farms A, B, and D and to the middle of June 2002 on farm C. On farm B, the bales weighed 650-750 kg (dimensions 1.2 x 1.2 x 2.4 m), and on farm D the bales weighed 250 kg (1.5 x 1.2 m round bales). Information about the bales on the other two farms was not provided. Windrows and bales were sampled in two

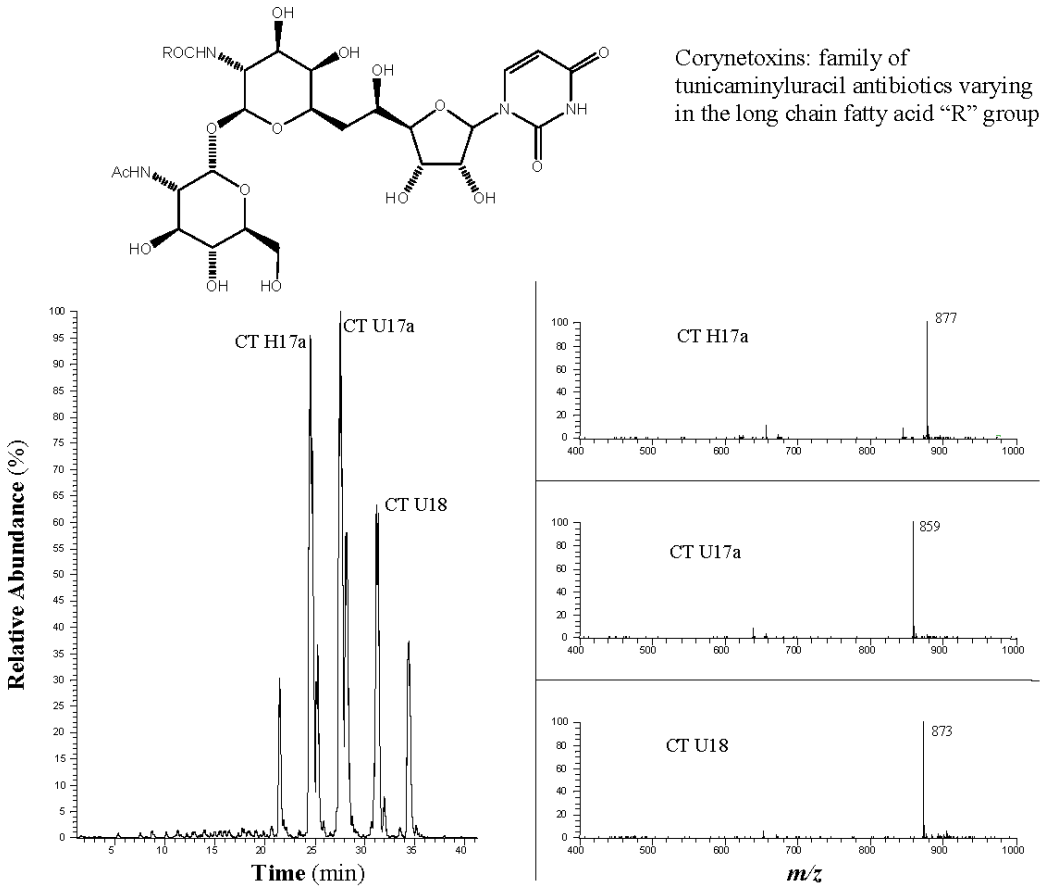

Figure 3. The general structure of the corynetoxins and a HPLC-esi(+)MS ion chromatogram of a corynetoxins sample showing, for example, the mass spectra for corynetoxins H17a, U17a, and U18.

paddocks on farm A, one paddock on farm B, four paddocks on farm C, and two paddocks on farm D. Samples of ryegrass heads from uninfected farm A for a spiking experiment were collected from along fence lines as for growing ryegrass at 14-day intervals from early September to mid-December 2001, providing a total of 8 samples (approximately 200 g each). On each farm, the repeat sample collections were taken from the same fence lines, the same windrows, or the same bales, as appropriate.

Ryegrass and hay samples were transferred to a cooler at 4 °C within an hour after collection and kept cool until delivery to the laboratory either in the afternoon the same day or early morning the next day. On arrival in the laboratory, the samples were kept at 4 °C until late afternoon (the same day or the next day), when they were soaked in 1% cyclodextrin to extract the *R. toxicus* antigen and the corynetoxins (*vide infra*). The soak extracts were stored at -15 °C ± 5 °C until the day of assay. All these samples were analyzed only in the Department of Agriculture and Food (DAFWA) laboratory using the described *R. toxicus* (Masters et al. 2006) and corynetoxins (Than et al. 2002) ELISAs.

Figure 4. (top) Driving corer into hay bale and (bottom) corer with hay sample in bag. Courtesy of Leo den Hollander.

Sample Extraction

All samples in this study were extracted by soaking overnight (approximately 17 h) in 5 volumes (v/w) of 1% methyl-β-cyclodextrin (Wacker Chemicals) in water in a tough plastic bag (figure 5). At the end of the soaking period, each sample was mixed thoroughly by kneading before cutting a corner of the bag to collect a mid-stream aliquot (1.5 mL) of the extract (to minimize the collection of soil or debris in addition to the clear extract). The soak extract samples were stored at −20 °C until tested.

Figure 5. Soaking hay samples (each plastic bag contains 1 kg hay plus 5 L of 1% methyl-β-cyclodextrin in water). Courtesy of Leo den Hollander.

The R. toxicus *Antigen ELISA*

The *R. toxicus* antigen ELISA (*R. toxicus* ELISA) used in these studies was performed as described by Masters et al. (2006) except that samples were extracted as above. The presence of 1% methyl-β-cyclodextrin does not affect the *R. toxicus* ELISA results (Masters, unpublished 2001). Semi-quantitative estimations of the amount of antigen present were performed as follows: Samples with an absorbance at 450 nm (Abs_{450}) reading higher than 2.0 when tested undiluted were tested in serial 10-fold dilutions (from 1 in 10 to 1 in 10,000, or higher if required) in 0.02 M phosphate buffered saline (pH 7.2) with 0.05% Tween 20 (Sigma P-1379) (PBST). The positive reference sample for this assay was an aqueous extract of contaminated oaten hay from the Wongan-Ballidu district of WA, containing bacterial galls as determined by identifying galls over a light box. The negative reference sample was an aqueous extract from ryegrass hay collected from Bedfordale (32.172°S, 116.047°E), an area where ARGT has not been recorded and that contained no bacterial galls. Both reference samples had been prepared when the ELISA was first developed and stored in1 mL aliquots at <−10 °C until used in assays. The positive reference was diluted as described (Masters et al. 2006). The ELISA results for each plate were accepted if the Abs_{450} for the positive reference was between 1.8 and 2.6 and the Abs_{450} for the negative reference was less than 0.25. For each sample dilution series from 1 in 10 to 1 in 10,000, the result chosen for calculating ELISA Units (EU) was that which fell within the linear range of the response curve (between 0.2 and 2.0); and if more than one result fell in this range, the result closest to the middle of the linear response curve was used (Masters et al. 2014). The EU calculation was simplified: [(Test Abs_{450} – Neg. reference Abs_{450}) x 100] x (reciprocal of the dilution for the sample). Experiments involving spiking of hay samples with bacterial galls have shown that using this simplified EU calculation, the relationship between EU and galls/kg is such that 10, 100, 1,000, 10,000, and 90,000 EU are equivalent to about 0.3, 1.7, 9.5, 52, and 300 galls/kg, respectively (Gregory, unpublished). The limit of sensitivity for this ELISA is 20 EU. The measurement uncertainty of the ELISA as determined at the DAFWA laboratory by repeated analysis of the same sample on different days and by different operators over a period of several months, during which different reagent batches were used, is 13% (coefficient of variation) at a mean value of 20 EU (n = 223) and 8.1% (n = 100) at a mean value of 5,943 EU.

The Corynetoxins ELISA

A quantitative indirect competitive ELISA for corynetoxins was developed by Than et al. (2002). The corynetoxins ELISA reagents were developed at the CSIRO and are not commercially available. Briefly, 96-well flat-bottom microtiter plates (Nunc 439454 Maxisorp) were pretreated with 0.2% glutaraldehyde in pH 9 carbonate buffer for 2 h at 56 °C. Plates were washed with pure water and then coated with chemically modified tunicamycins (4 ng/well in 100 µL pure water) for 2 h at 56 °C. The latter were prepared by treating commercially acquired tunicamycins (Fluka Analytical) with trifluoroacetic acid anhydride in trifluoroacetic acid, followed by hydrolysis to afford the diaminotunicaminyluracil derivative used for directly coating the glutaraldehyde-treated microtiter plates (Than et al. 1998). After washing 4 times with normal saline containing 0.5% Tween 20, dilutions of sample extracts in assay buffer (phosphate buffered saline pH 7.3 containing 0.5% protease-free bovine serum albumin and 0.05% Tween 20) and corynetoxin standards in 0.1% methyl-β-cyclodextrin in assay buffer were added, 100 µL/well in quadruplicate, followed by 50 µL sheep anti-corynetoxin antibodies (diluted 1 in

12,000 in assay buffer). The plate was mechanically shaken so that the contents of each well were mixed and then incubated at room temperature for 2 h. The wells were again washed 4 times with normal saline containing 0.5% Tween 20 and then incubated with anti-sheep IgG conjugated to horseradish peroxidase in assay buffer for 1 h at 37 °C. After washing again in the same way, the wells were finally incubated with 3,3'5,5'-tetramethyl benzidine substrate (K Blue, Cat. No. ESKE 1000, from ELISA Systems) for 30 min at room temperature. The color reaction was stopped by adding 50 µL/well of 0.5 M sulfuric acid, and the Abs_{450} was read. A standard curve was constructed by plotting the average Abs_{450} against the amount of standard corynetoxins added, and the resulting curve was used to determine the amount of corynetoxins in the diluted samples. Test samples were initially diluted 1 in 10; then samples with results that fell above the highest point on the standard curve were tested again at 1 in 100 in 0.1% methyl-β-cyclodextrin. The limit of quantitation for corynetoxins in hay with this ELISA was determined by Than et al. (2002) to be 40 µg/kg. The measurement uncertainty of this ELISA, determined at the DAFWA laboratory by repeated analysis of the same sample on different days and by different operators over a period of several months, during which different reagent batches were used, is 12% (coefficient of variation) at a mean value of 136.4 µg/kg (n = 172). Throughout this study, values below the level of quantitation were entered into data analysis spreadsheets as the values recorded (not as zero).

Inter-Laboratory Study

In 2001, an inter-laboratory study was conducted to ensure that the *R. toxicus* and corynetoxin ELISAs met acceptable standards for the quality assurance (QA) of hay in terms of reproducibility within and between laboratories and the robustness of the assays. The participating laboratories were the Animal Health Laboratories at DAFWA, the CSIRO Plant Toxins Research Group at the Australian Animal Health Laboratories, and the Plant Research Centre at the South Australian Research and Development Institute (SARDI). Soak extracts from 100 hay samples were made at SARDI (samples 1-10) and DAFWA (samples 11-100), each divided into 1.2 mL aliquots and stored frozen until 1 aliquot of each extract was dispatched in a polystyrene insulated container with ice packs to each of the 3 participating laboratories. The laboratories performed the *R. toxicus* ELISA with reagents and procedures supplied by DAFWA (Masters et al. 2006), and the corynetoxins ELISA with reagents and procedures supplied by CSIRO (Than et al. 2002) on the same aliquot, stored frozen between assays.

Comparison Methods and Statistical Analysis for Inter-Laboratory Study

The data obtained by the three laboratories were analyzed at DAFWA in five ways.

(A) *R. toxicus* ELISA versus corynetoxins ELISA in each laboratory. First, it was determined how many samples each laboratory categorized as "higher" (>90,000 EU) and "lower" (<90,000 EU) by the *R. toxicus* ELISA. The value 90,000 EU was chosen because this level is just below the mean (but above the median) found in 14 samples spiked with 100 bacterial galls/kg in a previous study (Masters et al. 2014). The two categories of *R. toxicus* ELISA results were each further divided into four corynetoxins result categories, based on the average corynetoxins ELISA result from the three laboratories, such that each category contained roughly one quarter (20-30) of the samples. The first category, 0 – <40 µg/kg, was all results below the limit of quantitation. The other three categories were 40 – <100 µg/kg, 100 – <400 µg/kg, and ≥400 µg/kg. The number of "higher" or "lower" *R. toxicus* results in each corynetoxins result category was then determined for each laboratory.

(B) Reproducibility of each assay. The coefficient of variation (CV) of the difference in results between the three laboratories was calculated as the between laboratory standard deviation expressed as a percentage of the mean for each assay.

(C) Inter-laboratory agreement. The results were divided into three categories: high, medium, and low, each category containing roughly one-third of the results. Each pair of laboratories (DAFWA and SARDI, DAFWA and CSIRO, and CSIRO and SARDI) was then compared on the basis of the number of samples falling into the categories high (*R. toxicus* antigen >183,000 EU; corynetoxins >180 µg/kg), medium (*R. toxicus* antigen 38,000 to 183,000 EU; corynetoxins 60 to 180 µg/kg), and low (*R. toxicus* antigen <38,000 EU; corynetoxins <60 µg/kg).

(D) Inter-laboratory correlation. The correlation coefficients between results from pairs of laboratories were calculated for each ELISA.

(E) Correlation between *R. toxicus* ELISA and corynetoxins ELISA results. The correlation between results for the two ELISAs obtained within each laboratory was calculated, and an overall correlation between the average *R. toxicus* EU and the average corynetoxins concentrations for each sample obtained by the three laboratories

was calculated. These correlations were also calculated after natural logarithm transformation to achieve a closer to normal distribution. A constant of 10,000 was added to *R. toxicus* before the log transformation was applied.

Statistical Analysis for Samples Collected From the Field

Samples were collected from the field to examine the association between the *R. toxicus* antigen and corynetoxins as assessed by the ELISAs when testing (i) infected, standing ryegrass over its normal growth and maturation cycle; (ii) ryegrass pasture after cutting, baling, and storage in sheds; and (iii) an experiment with spiked ryegrass samples. The correlation between *R. toxicus* antigen and corynetoxin concentrations was calculated for each of the three datasets. The spiked samples experiment was performed with samples collected on uninfected farm A. The samples were split equally into two with one subsample being kept as a reference sample and the other having five bacterial galls added to it to create a spiked sample. The bacterial galls were randomly picked from bacterial galls identified over a light box in 1999. An overall correlation between the *R. toxicus* EU and the corynetoxins concentrations in all the samples from the field was calculated.

After collection of all the data for the above studies, it was found that DAFWA used the ProSciTech brand of glutaraldehyde to prepare wells of the corynetoxins ELISA plates for coating with modified tunicamycin, whereas CSIRO used the AJAX brand. The two brands were compared on the same type of ELISA plates on the same day with the same reagents and the same batch of samples (that were negative in the *R. toxicus* ELISA) at DAFWA. The significance of the difference between the two sets of corynetoxins results was calculated using a paired t-test.

Results

Inter-Laboratory Study

Table 1 shows the results obtained by the three laboratories after analyzing 100 hay sample extracts for the bacterial antigen and the corynetoxins.

Table 1. *R. toxicus* antigen and corynetoxins ELISA results as determined at three laboratories: DAFWA (D), CSIRO (C), and SARDI (S)

Sample No.	*R. toxicus* antigen (ELISA Units x 10^{-2})					Corynetoxins (µg/kg)				
	D	C	S	3-Lab Mean	CV (%)	D	C	S	3-Lab Mean	CV (%)
1	50	49	70	56	21	63	41	15	40	61
2	18,805	10,602	14,690	14,699	28	989	1,335	916	1,080	21
3	5,935	1,782	1,379	3,032	83	384	499	606	497	22
4	0.2	0.1	0.1	0.1	16	47	50	17	38	48
5	0.1	0.1	0.0	0	42	68	65	12	48	65
6	0.1	0.1	0.0	0	58	77	75	41	64	31
7	10,495	12,377	11,460	11,444	8	323	402	527	417	25
8	1,467	802	790	1,019	38	423	350	146	306	47
9	0.8	0.5	0.6	1	30	105	58	42	68	48
10	19,670	12,887	18,400	16,986	21	1,496	1,921	1,271	1,563	21
11	15,920	7,762	6,520	10,067	51	3,890	3,201	2,405	3,165	23
12	12,835	8,512	7,360	9,569	30	1,561	1,920	1,746	1,743	10
13	16,650	7,182	6,240	10,024	57	2,531	2,497	2,157	2,395	9
14	4,405	1,755	2,820	2,993	45	894	938	1,179	1,003	15
15	12,600	1,510	4,060	6,057	96	2,466	1,965	2,482	2,304	13
16	74	77	90	80	10	91	81	26	66	53
17	37	17	17	24	48	58	43	24	42	41
18	8,780	1,698	3,560	4,679	78	395	422	248	355	26
19	0.5	0.2	0.4	0.4	45	64	56	11	44	65
20	4,800	1,710	3,140	3,217	48	514	542	403	486	15
21	1,647	892	884	1,141	38	146	171	119	145	18
22	744	472	518	578	25	104	86	71	87	19
23	172	151	153	159	7	99	83	42	75	39
24	10,135	1,308	0.5	3,814	145	2,436	2,807	2,286	2,510	11

25	118	106	148	124	17	40	35	37	37	7
26	16,395	8,217	11,750	12,121	34	318	385	403	369	12
27	18,500	18,417	15,040	17,319	11	908	933	1,128	989	12
28	1,204	1,607	1,749	1,520	19	164	127	97	129	26
29	3,005	1,875	2,104	2,328	26	106	64	67	79	30
30	14,825	9,162	6,140	10,042	44	524	560	512	532	5
31	935	1,043	859	945	10	194	153	154	167	14
32	16,095	12,772	10,370	13,079	22	381	211	249	280	32
33	107	141	155	134	18	31	15	15	20	44
34	1,692	1,546	1,485	1,574	7	193	143	155	163	16
35	5,395	5,327	3,100	4,607	28	1,731	1,840	1,607	1,726	7
36	18,695	16,737	12,610	16,014	19	2,274	2,789	2,193	2,418	13
37	4,495	4,037	2,960	3,831	21	531	602	562	565	6
38	18,295	15,477	13,210	15,661	16	3,364	4,546	3,060	3,656	21
39	4,005	6,287	154	3,482	89	653	812	598	688	16
40	0.9	5.0	6.7	4	70	27	23	12	21	37
41	380	354	273	335	17	120	67	56	81	42
42	0.5	0.3	0.3	0	24	36	26	18	26	35
43	6.0	0.9	81.1	29	153	38	30	18	29	36
44	0.8	0.5	0.6	1	22	40	25	14	26	50
45	0.2	0.1	0.1	0.1	50	34	35	19	29	30
46	0.7	0.4	0.6	1	27	69	50	36	52	32
47	38	800	376	404	94	37	35	16	30	39
48	3,025	2,022	1,373	2,140	39	188	154	159	167	11
49	6	6	8	7	19	36	39	19	31	33
50	0.1	0.1	0.0	0	39	42	47	34	41	16
51	0.1	0.0	0.0	0	60	37	40	19	32	35
52	13	14	27	18	42	32	2	9	15	107
53	0.3	0.1	0.2	0.2	33	30	20	16	22	33
54	30	49	25	35	36	40	21	20	27	41
55	1,085	877	436	799	41	64	48	37	50	27
56	7,065	1,959	1,547	3,524	87	211	120	143	158	30
57	1,138	1,544	974	1,218	24	93	56	69	73	26
58	1,097	1,247	608	984	34	90	51	48	63	37
59	1,553	1,593	899	1,348	29	193	120	115	143	31
60	7,885	2,040	1,645	3,857	91	302	193	192	229	28
61	3,715	1,483	689	1,962	80	112	75	73	87	25
62	8,485	11,177	6,120	8,594	29	483	708	494	561	23
63	1,708	1,333	838	1,293	34	132	110	138	126	11
64	65	151	49	88	62	36	32	42	36	14
65	9,715	7,417	3,460	6,864	46	580	449	516	515	13
66	1,864	1,617	1,020	1,500	29	173	120	143	145	18
67	1,833	1,592	924	1,449	32	166	111	149	142	20
68	158	756	482	465	64	89	54	64	69	27
69	2,545	11,827	5,760	6,711	70	846	1,046	778	890	16
70	269	895	471	545	59	100	54	43	66	45
71	917	4,677	3,130	2,908	65	398	319	324	347	13
72	2,585	6,987	3,940	4,504	50	700	528	493	574	19
73	4	17	32	17	82	49	11	19	27	76
74	431	1,605	1,193	1,076	55	170	179	181	176	3
75	178	1,128	873	726	68	117	97	91	102	14
76	369	195	275	280	31	67	33	39	46	38
77	497	574	346	472	25	59	54	57	56	4
78	1,602	1,375	878	1,285	29	149	112	122	128	15
79	126	103	315	181	64	44	40	38	40	8
80	1,609	5,500	4,620	3,910	52	122	76	100	99	23

81	1,391	1,809	4,300	2,500	63	121	96	100	106	13
82	521	558	852	643	28	40	46	21	36	36
83	1,072	1,575	1,700	1,449	23	98	104	81	94	13
84	1,371	1,250	1,389	1,336	6	139	157	128	142	10
85	6,375	5,595	7,020	6,330	11	590	508	493	530	10
86	14,660	16,745	21,720	17,708	20	1,275	1,573	1,043	1,297	20
87	1,727	3,525	3,460	2,904	35	143	143	124	137	8
88	3,810	5,205	6,690	5,235	28	308	341	291	314	8
89	286	440	663	463	41	64	50	34	49	31
90	1,038	1,395	1,831	1,421	28	128	118	106	118	9
91	949	1,239	1,693	1,294	29	86	75	69	77	11
92	469	591	1,019	693	42	64	84	73	73	13
93	533	733	975	747	30	55	40	28	41	34
94	1,687	1,791	3,290	2,256	40	186	151	136	158	16
95	302	343	549	398	33	66	54	42	54	22
96	377	484	736	532	35	90	64	55	70	26
97	242	168	440	283	50	55	35	28	39	36
98	38	49	76	54	37	36	43	33	37	14
99	2,220	3,845	3,950	3,338	29	144	135	116	132	11
100	1,510	1,886	3,570	2,322	47	97	121	109	109	11

(A) *R. toxicus* ELISA vs corynetoxins ELISA in each laboratory (table 2). There were 24 extracts for which the mean of the three laboratories showed a high corynetoxins concentration (>400 µg/kg), and two of the laboratories identified all these extracts as "higher" according to the *R. toxicus* ELISA. The third laboratory identified 22 of the 24 extracts as "higher" according to the *R. toxicus* ELISA. All laboratories identified the 21 extracts with the lowest corynetoxins concentrations (0–<40 µg/kg) as "lower" according to the *R. toxicus* ELISA. Most of the 27 extracts with corynetoxins concentrations between 100 and <400 µg/kg were identified as "higher," while most of the 28 extracts with corynetoxins concentrations between 40 and <100 µg/kg were identified as "lower" in the *R. toxicus* ELISA by each laboratory.

(B) Reproducibility of each assay. The average between laboratory CV for the *R. toxicus* ELISA was 41.6% (SD 26.4, range 6-153), while for the corynetoxins ELISA it was 25.4% (SD 17.0, range 3-107). Analysis of variance showed a significant difference in CV between the assays ($p<0.001$) indicating better reproducibility for the corynetoxins ELISA.

(C) Inter-laboratory agreement (tables 3a-e). For both the *R. toxicus* ELISA and the corynetoxins ELISA, there was high consistency between laboratories in terms of the broad categorization of high/medium/low. For the corynetoxins ELISA, there were no instances where one laboratory gave a high result (top third) and another laboratory gave a low result (bottom third), while for *R. toxicus* ELISA, this happened three times (1% of comparisons).

(D) Inter-laboratory correlation. For the *R. toxicus* ELISA, the correlations between laboratories were DAFWA and SARDI r = 0.86, DAFWA and CSIRO r = 0.85, and SARDI and CSIRO r = 0.92. For the corynetoxins ELISA, the correlations between laboratories were DAFWA and SARDI r = 0.98, DAFWA and CSIRO r = 0.97, SARDI and CSIRO r = 0.98. All correlations for both analytes were highly significant ($p<0.001$). An observation, however, was that the corynetoxins ELISA results obtained at DAFWA were higher than the results of the other two laboratories for 59 samples out of 100, and this tendency was even more pronounced when results below 100 µg/kg were compared: DAFWA result was higher in 33 out of 41 (80%).

(E) Correlation between *R. toxicus* ELISA and corynetoxins ELISA results. The correlations found between *R. toxicus* ELISA results and corynetoxins ELISA results within each laboratory were DAFWA r = 0.75, CSIRO r = 0.64, and SARDI r = 0.57. The overall correlation between the average *R. toxicus* EUs and the average corynetoxins concentrations for each sample obtained by the three laboratories was r = 0.70. The correlations on the log transformed data were DAFWA r=0.88, CSIRO r=0.81, SARDI r=0.80, and Average r=0.87. Again, all correlations were highly significant ($p<0.001$).

At the low end of the scale for both analytes, taking the average of the results from the three laboratories for each analyte, there were detectable quantitation in 16 samples (table 1 numbers 25, 33,

Table 2. Number of extracts classified as higher according to the *R. toxicus* ELISA, in each of four categories based on corynetoxin concentrations, for each of the three laboratories

		Number of samples			
		Corynetoxins, µg/kg (average of three labs)			
		0-<40	40-<100	100-<400	≥400
DAFWA	higher (*R. toxicus* > 90,000 EU)	0	8	25	24
	lower (*R. toxicus* < 90,000 EU)	21	20	2	0
CSIRO	higher (R. toxicus >90,000EU)	0	7	25	24
	lower (R. toxicus < 90,000 EU)	21	21	2	0
SARDI	higher (*R. toxicus* > 90,000 EU)	0	7	20	22
	lower (*R. toxicus* < 90,000 EU)	21	21	7	2

Table 3. Between laboratory agreement for *R. toxicus* and corynetoxins ELISAs

(a) CSIRO v DAFWA

R. toxicus	DAFWA low[1]	DAFWA medium[1]	DAFWA high[1]
CSIRO low	30	1	0
CSIRO medium	6	23	9
CSIRO high	0	4	27

(80% correct match, 0% high-low)

(b) CSIRO v DAFWA

Corynetoxin	DAFWA low[2]	DAFWA medium[2]	DAFWA high[2]
CSIRO low	24	12	0
CSIRO medium	0	26	7
CSIRO high	0	0	31

(81% correct match, 0% high-low)

(c) CSIRO v SARDI

R. toxicus	SARDI low	SARDI medium	SARDI high
CSIRO low	29	2	0
CSIRO medium	3	29	6
CSIRO high	1	3	27

(85% correct match, 1% high-low)

(d) CSIRO v SARDI

Corynetoxin	SARDI low	SARDI medium	SARDI high
CSIRO low	34	2	0
CSIRO medium	6	26	1
CSIRO high	0	1	30

(90% correct match, 0% high-low)

(e) SARDI v DAFWA

R. toxicus	DAFWA low	DAFWA medium	DAFWA high
SARDI low	29	2	2
SARDI medium	7	20	7
SARDI high	0	6	27

(76% correct match, 2% high-low)

(f) SARDI v DAFWA

Corynetoxin	DAFWA low	DAFWA medium	DAFWA high
SARDI low	24	16	0
SARDI medium	0	21	8
SARDI high	0	1	30

(75% correct match, 0% high-low)

[1]The categories for *R. toxicus* ELISA are <38,000 EU (low), 38,000 to 183,200 EU (medium), and >183,200 EU (high).
[2]The categories for Corynetoxin ELISA are <60 µg/kg (low), 60 to 180 µg/kg (medium), and >180 µg/kg (high).
The categories for both assays were chosen on the basis of one-third of the samples falling in each category and do not necessarily reflect risk (of ARGT) levels.

40, 42, 43, 44, 47, 49, 52, 53, 54, 64, 73, 82, 97, and 98), *R. toxicus* levels but corynetoxins concentrations below the limit of but still all in the lower half of the dataset with *R. toxicus* levels below 65,000. There were detectable corynetoxins but *R. toxicus* levels below the limit of quantitation in three samples (table 1 numbers 5, 6, and 50); however, for these three samples, the level of corynetoxins was still very low (<100). There were also three samples with both *R. toxicus* levels and corynetoxins concentrations below the limit of quantitation (table 1 numbers 4, 45, and 51).

Samples Collected From the Field in WA

(i) Infected ryegrass over its normal growth and maturation cycle. Compliance with sample collection instructions for this section was generally poor: sample weights ranged from 1 g to 225 g. The time between consecutive sample

collections became longer in the 2 months after cutting (late October) and baling (first week of November). The correlation between *R. toxicus* antigen and corynetoxins concentrations in ryegrass heads in these samples was r = 0.79 ($p<0.001$, n = 252).

(ii) Correlation of the bacterial and toxin antigens in pasture after cutting for hay. Compliance with the sample collection instructions for this part of the field study was good on farms A and B, where staff followed the export industry protocol, except that sampling from windrows was infrequent on farm A. The number of days between cutting and baling was approximately 24, 7, 26, and 12 on farms A, B, C, and D, respectively. Samples from the windrows on farm A varied from 800 g to 1,000 g (mean 967 g, n = 6), and samples from the bales ranged from 106 g to 296 g (mean 204 g). The correlation between *R. toxicus* antigen and corynetoxins concentrations for this experiment on cut pasture before and after baling was r = 0.77 ($p<0.0001$, n = 121).

(iii) Spiked ryegrass samples. Results for the eight reference samples were all below the respective limits of quantitation of the two ELISAs. The *R. toxicus* ELISA results for the 8 spiked samples ranged from 8125 to 78,800 EU (average 48,622 EU), and the corresponding corynetoxins results ranged from 18 to 65 µg/kg (average 49.6 µg/kg, only one result being below 40, the limit of quantitation). The correlation between the *R. toxicus* and corynetoxins results in this experiment was r = 0.87 ($p<0.001$, n = 16).

In the samples collected from the field, including the spiked samples, the overall correlation between results obtained with the *R. toxicus* ELISA and the corynetoxins ELISA (figure 6) was r = 0.80 ($p<0.001$, n = 389). At the low end of the scale for both analytes, there were detectable *R. toxicus* levels but corynetoxins concentrations below the limit of quantitation in 92 samples, although none of these had *R. toxicus* greater than 100,000 EU. There were also 23 samples with detectable corynetoxins but *R. toxicus* levels below the limit of quantitation, but these all had very low corynetoxin concentration (<150). There were 152 samples with both *R. toxicus* levels and corynetoxins concentrations below the limit of quantitation. The relationship at the lower end of the scale can be seen better when the data are plotted on the log scale (figure 7).

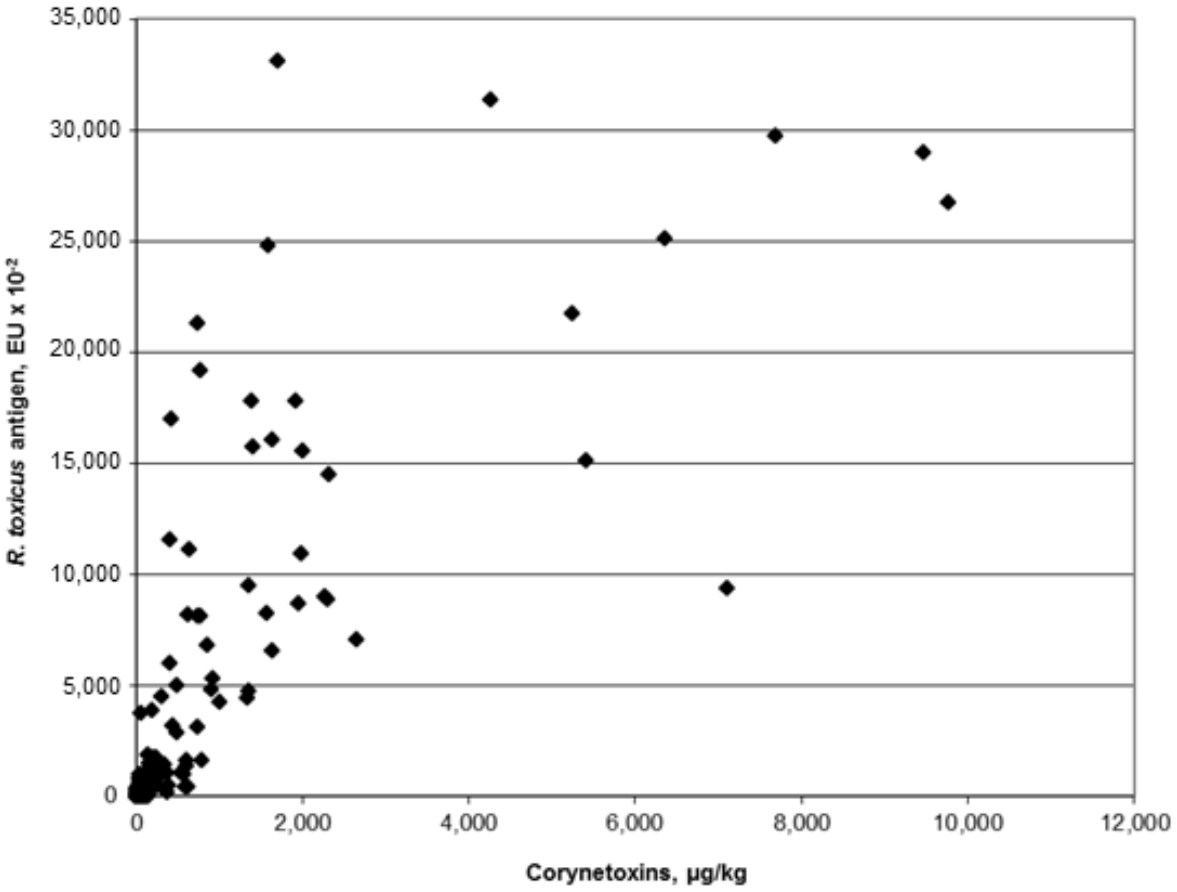

Figure 6. Scatter diagram showing the relationship between *R. toxicus* antigen and corynetoxins observed in all samples (n = 389) collected from the field in Western Australia.

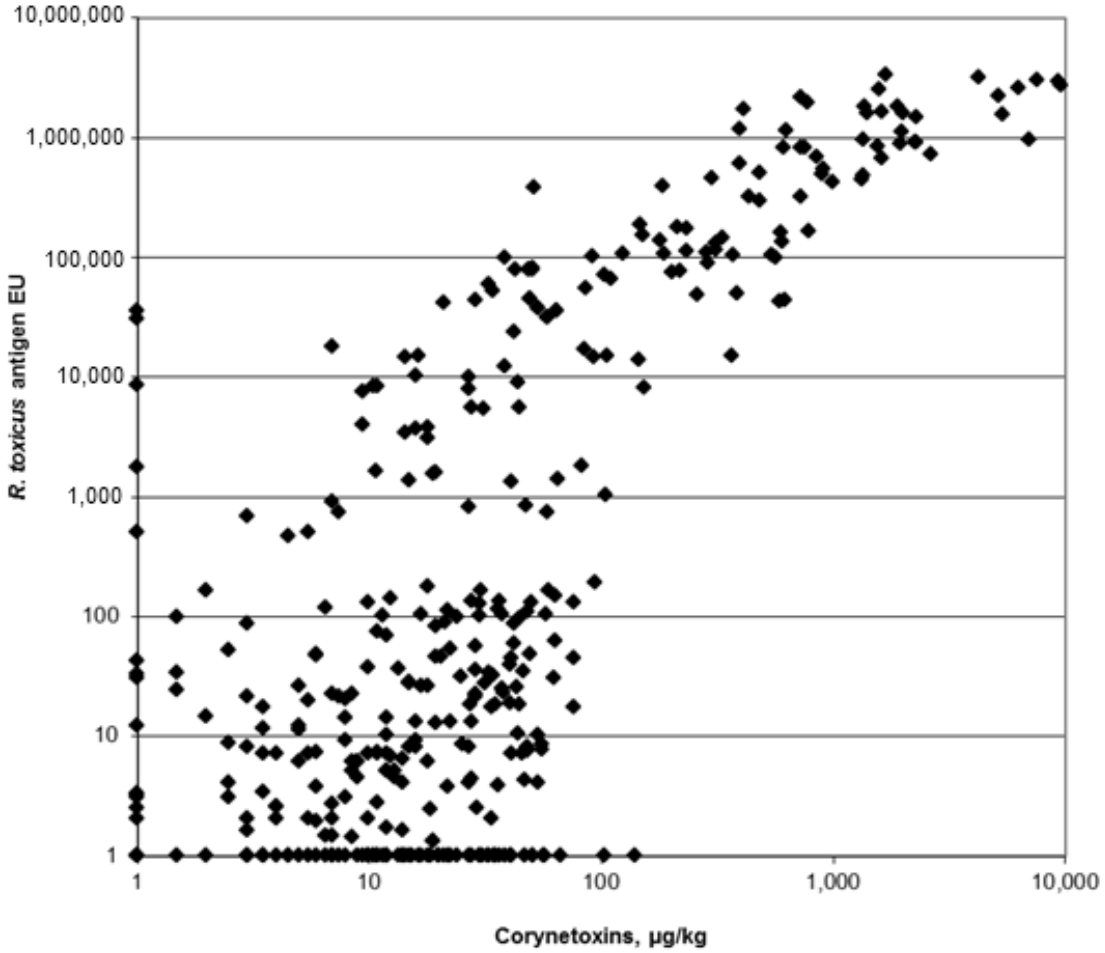

Figure 7. Scatter diagram showing results for *R. toxicus* antigen and corynetoxins on a log scale, in samples collected from the field in Western Australia (n = 389). So that the many zero results were included on the log scale, all values less than 1 were plotted as 1.

When tested on plates coated after pre-treatment with ProSciTech glutaraldehyde, the mean corynetoxins result for a set of samples with negative results for *R. toxicus* was 57 µg/kg (SD = 14.7, n = 19), compared with a mean of 32 µg/kg (SD = 8, n = 19) on plates pre-treated using AJAX glutaraldehyde. This difference was significant by a paired t-test ($p<0.001$) and was unexpected (see discussion).

Discussion

Throughout both studies, samples were stored for up to 2 days before extraction by soaking in 1% cyclodextrin. It is possible that during storage, toxin production may continue inside the galls, particularly when moisture is still present; and this may possibly occur in the early stages of soaking when galls take up water. It is not known how much additional toxin would be elaborated during 2 days of storage or during soaking (if this occurs). Further studies may be required.

In the inter-laboratory study, the results of comparison method A indicated that all three laboratories generally found that when the *R. toxicus* ELISA result was high, the corynetoxins result was also high, and when the *R. toxicus* ELISA result was low, the corynetoxins result was also low. The corynetoxins ELISA was more reproducible than the semi-quantitative *R. toxicus* ELISA (comparison method B). The features that potentially reduce variability of the corynetoxins ELISA between laboratories are (1) there is a standard curve on every plate, (2) all samples and standards are assayed in quadruplicate, and (3) there is only a single dilution of each sample. Probable explanations for the greater variation in the *R. toxicus* ELISA would include the performance of the serial 10-fold dilutions performed in the ELISA plate (differences between operators and between pipettes) because any error is compounded in successive dilutions. Additionally, when for some samples, the absorbance result for the calculation falls in a non-linear section of the antigen/absorbance response curve, the variation is greater than when it falls in the linear range. The above explanations, however, do not account for the disparity in results seen for 12 samples (numbers 3, 15, 18, 24, 39, 43, 47, 56,

60, 61, 73, and 75 in table 1). It is possible that for these samples, there were errors such as inadvertent sample substitution or calculation error (such as entering the wrong dilution or failing to multiply by the reciprocal of the dilution) or failing to notice that precipitation of the substrate had occurred before reading the ELISA plate. Precipitation can occur in wells with high levels of antigen and will reduce the measured OD by variable amounts depending on the degree of precipitation at the time of reading the plate. Comparison method C indicated good agreement between the laboratories with 81% of comparisons giving the same ranking of high/medium/low, and no high-vs-low discrepancies for the corynetoxin ELISA. Comparison method D showed highly significant positive correlations between laboratories for both ELISAs, the correlations being higher for the corynetoxins ELISA. Comparison method E demonstrated that within each laboratory, a highly significant positive correlation between *R. toxicus* ELISA results and corynetoxins ELISA results was found. Also, when the results of each analyte from all three laboratories were averaged for each sample, there was a highly significant positive correlation between the average *R. toxicus* antigen ELISA results and the average corynetoxins ELISA results. Overall, the inter-laboratory study showed that both ELISAs are robust enough to be performed reliably in different laboratories, as well as showing a significant positive correlation between results of the two ELISAs.

The results of the spiking experiment in samples collected from the field showed that both the *R. toxicus* antigen and the corynetoxins concentrations, when determined by the ELISAs described in this paper, are indeed associated with bacterial galls. Other possible sources of toxin in ryegrass and hay samples are bacterial slime and fines. There was a highly significant correlation between the *R. toxicus* and corynetoxins ELISA results in this experiment. Bacterial galls can vary with respect to weight and the quantity of corynetoxins (Cockrum and Edgar 1985). The range of results seen in the spiked samples reflects variation in the amounts of both the bacterial antigen and corynetoxins between galls and possibly variation in extraction efficiency between samples.

There were 3 samples in the inter-laboratory study and 23 among the field samples for which low levels of corynetoxins were detected and *R. toxicus* antigen was not detected. It is quite possible that most of these are false positives. As mentioned in the Results section, the DAFWA corynetoxins results were generally higher than those of the other two laboratories for the samples at the low end of the range. The coating step of the corynetoxins ELISA requires pre-treatment of the wells with glutaraldehyde, and it was found after testing of the samples from the field in WA was completed that the brand of glutaraldehyde makes a difference to the results at the low end of the range. The DAFWA laboratory used a different brand (Pro Sci Tech) from CSIRO (Ajax), and this apparently raised the limit of quantitation in the DAFWA laboratory. This result was not expected because the Pro Sci Tech brand is of higher purity. In hindsight, glutaraldehyde of higher purity may cause greater “activation” of sites on the polystyrene surface of the wells and thereby promote binding of a higher proportion of the modified tunicamycin to the plate at the first step of the ELISA. Since it is a competitive ELISA, the amount of anti-corynetoxin antibody bound to the modified tunicamycin will be affected very little for the high corynetoxin standards, but it will be increased for the low corynetoxin standards. This would change the slope of the standard curve and thereby potentially cause false positive results.

There was a significant positive correlation between the results of the two ELISAs when they were performed on the same soak extracts of ryegrass or hay samples. Distribution and abundance of bacterial galls within a paddock can be highly variable. When assessing the suitability of pasture or hay for stock to graze, sampling strategies must ensure that the samples taken represent the whole paddock or bale. Before baling, this requires collecting at least 20 subsamples while walking in zigzag fashion across the paddock, then pooling the subsamples for testing. After baling, the procedures to follow have been described in detail (Elson 2002b). Even when the *R. toxicus* ELISA result is low, stock still need to be observed frequently for signs of ARGT, because there have been cases where only part of a paddock or just one bale was highly infected and caused stock deaths. In these situations, it is hard to track down the source of the corynetoxins.

In both the inter-laboratory study and the samples collected from the field, the correlation between *R. toxicus* antigen and corynetoxins was positive and highly significant. At the low end of the range of both analytes and in both studies, more samples were positive for *R. toxicus* and negative for corynetoxins than vice versa. The reasons may be that the *R. toxicus* ELISA is more sensitive and that log-phase bacterial multiplication precedes toxin production (Payne and Cockrum 1988). The measurement uncertainty of the assays may also be an important factor at the low end of the range. At the medium and high end of the range of results, we found that if *R. toxicus*

antigen was detected in hay or pasture samples, corynetoxins were also detected.

Implications/Recommendations

The *R. toxicus* ELISA can therefore be used as an indirect measure of corynetoxins in hay and pasture samples.

The *R. toxicus* ELISA has remained the method for export testing because it is better suited for high-throughput testing (42 samples per ELISA plate vs 10), it requires only water for extracting the antigen in soak extracts of the hay samples, and it is very sensitive. In addition, the hay export *R. toxicus* ELISA is on undiluted extracts and for this reason is very robust (no chance of dilution errors or calculation errors).

If no *R. toxicus* antigen is detected by the *R. toxicus* ELISA in a representative sample of hay, then there should be little or no risk of poisoning.

The corynetoxins ELISA is a potentially useful research tool but is not practical for routine testing. When the corynetoxins ELISA is performed, the limit of quantitation should be re-evaluated for the brand/batch of glutaraldehyde in use.

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Gastrolobium spp. Poisoning in Sheep—A Case Report

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Abstract

This report describes the history and investigation of a suspected plant poisoning event in Western Australia where 15 sheep died. One of the poisoned sheep was necropsied, and gross and microscopic pathology of the poisoned sheep is described. Monofluoroacetate was detected in rumen contents from the necropsied sheep. The case history, pathological changes, and detection of monofluoroacetate in the rumen contents support a diagnosis of monofluoroacetate intoxication. A review of the literature suggests this is the first example of detection of monofluoroacetate in the rumen contents of an animal poisoned by a plant containing monofluoroacetate.

Keywords: *Gastrolobium* spp., monofluoroacetate, poisoning, sheep

Introduction

Many plants found primarily in the southern continents of Africa, Australia, and South America, belonging to the Fabaceae, Rubiaceae, Bignoniaceae, Malpighiaceae, and Dichapetalaceae families, contain monofluoroacetate and adversely affect livestock production on these continents (Lee et al. 2014). Australian species of *Gastrolobium* and *Acacia* produce monofluoroacetate and have had a significant impact in the settlement of Australia (McKenzie 2012). Early colonists of Western Australia (WA) experienced heavy livestock losses due to animal consumption of *Gastrolobium* spp., which resulted in the colloquial term for the plants as "poison peas" (Marchant 1994). There are over 100 *Gastrolobium* spp., primarily in WA, and many continue to affect modern-day farming (Chandler et al. 2002, 2003).

Aplin (1971) reported large variations in monofluoroacetate concentrations between different *Gastrolobium* spp. and between plants in the same species even at the same location.

Monofluoroacetate concentrations tend to be highest in reproductive tissues such as pods, flowers, and young leaves and much lower in mature leaves and wood (Aplin 1971, Hall 1972, Twigg et al. 1996b, Twigg et al. 1999). There are varying degrees of evidence for toxicity in 39 species of *Gastrolobium* (Bennetts 1935, Gardner and Bennetts 1956, Gardner 1964, Aplin 1971, Twigg et al. 1996a,b, Twigg et al. 1999, Chandler et al. 2002) and an additional 7 species are suspected or presumed to be toxic (Chandler et al. 2002).

This report describes the history and investigation of a suspected plant poisoning event in WA. The gross and microscopic pathology of one of the poisoned sheep is described. Analysis of the rumen contents suggested that the ingestion of a toxic monofluoroacetate-containing plant (*Gastrolobium* spp.) was the probable cause of the poisonings. This appears to be the first report of detection of monofluoroacetate in the rumen contents of a poisoned animal being used to support a diagnosis that they have consumed a *Gastrolobium* sp.

History

In February 2013, 1,200 young mated Merino ewes were introduced into a pasture near Merredin, WA. On or about April 18, 2013, there was a rainfall event. The next day, 10 ewes were found dead. The owner decided to remove the remaining ewes by walking them out of the pasture. Several animals walked slowly, kept stopping, and became recumbent. One of the ewes that became recumbent subsequently died, for a total of 11 dead sheep in this incident. The owner left about 20 sheep in the pasture. The following May, there was another rainfall event, and the owner revisited the pasture the next day to find four dead sheep. A total of 15 sheep died in the two episodes.

Gross Pathology

A sheep in good post-mortem condition from the second episode was necropsied. The ewe was in good body condition and not pregnant. Petechiae were in the myocardium, kidneys were soft, and rumen contents were green and fluid in consistency. Most of the rumen contents were accidentally discarded, with only a few plant pieces saved for identification and analysis.

Suspected Cause of Death

Findings at necropsy suggested enterotoxemia, but the sheep had received two vaccinations against this disease. Also, two episodes of synchronous deaths immediately after rainfall are inconsistent with enterotoxemia, being more consistent with fluoroacetate poisoning, since after a rainfall sheep nibble on toxin-containing bushes. The attending veterinarian suspected that *Gastrolobium stenophyllum* (narrow-leaved poison) was available to these sheep.

Histopathology

Tissues collected at necropsy were submitted to the Animal Health Laboratories of the Department and Agriculture and Food Western Australia for microscopic examination. In heart sections, rare myofibers were swollen, hypereosinophilic, fragmented, and had pyknotic nuclei (necrosis) (figure 1). There was also hypertrophy of interstitial cells, diffuse congestion, and multiple hemorrhages. Lung sections were congested, and proteinaceous fluid filled airways. A light lymphocytic and plasmacytic, periportal infiltrate was in liver sections. No significant findings were observed in sections of brain.

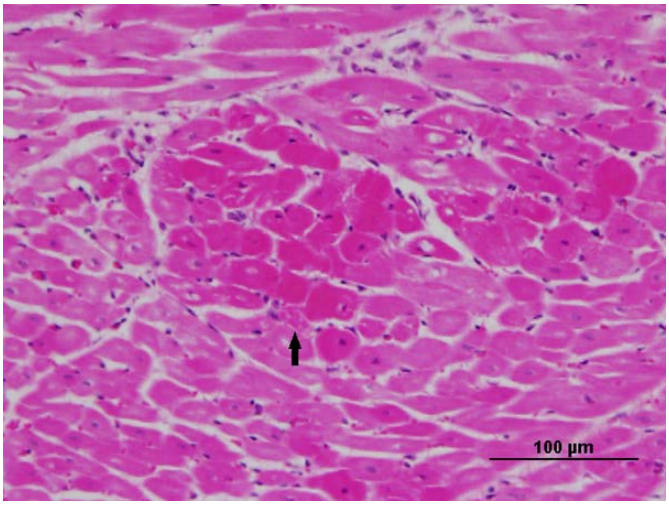

Figure 1. Heart (myocardium) with a focal area of necrosis. The necrotic myofibres are swollen, hypereosinophilic, and fragmented with pyknotic nuclei (arrow).

Mild, multifocal, acute, myocardial necrosis, together with moderate, diffuse pulmonary edema, are suggestive of cardiac toxicosis. In WA, the ingestion of plants containing either monofluoroacetate or cardiac glycosides is usually suspected as the cause of such changes in ruminants that have died unexpectedly.

Plant Identification

Plant fragments recovered during necropsy were examined and could not be positively identified as from a *Gastrolobium* spp.

Chemistry

Using a HPLC-APCI-MS method developed by Lee et al. (2012), monofluoroacetate was detected in the plant material collected from the rumen during necropsy at a concentration of 63mg/kg (figure 2). The identification of monofluoroacetate-containing plant material in the rumen at the time of death supported a diagnosis of monofluoroacetate poisoning, and the veterinarian's suspicion that the

plant fragments collected were from a *Gastrolobium* spp.

Summary

In conclusion, the case history, pathological changes, and detection of monofluoroacetate in rumen contents supported a diagnosis of monofluoroacetate intoxication. A review of the literature suggests that this is the first example of detection of monofluoroacetate in rumen contents of an animal poisoned by a plant containing monofluoroacetate. Methods have been developed to detect monofluoroacetate in rumen contents and liver samples. Monofluoroacetate was spiked into the liver or gastric contents, and it was determined how much could be recovered (Minnaar et al. 2000). In addition, monofluoroacetate has been detected in the kidneys of a lamb and a ewe diagnosed with 1080 (monofluoroacetate) poisoning (Giannitti et al. 2013), and monofluoroacetate has been detected in the blood, heart, skeletal muscle, and liver of sheep that died due to experimental poisoning with 1080 (monofluoroacetate). Significantly, in the last situation, monofluoroacetate was not detected in any of the organs of animals that survived (Gooneratne et al. 2008). In none of these examples was plant material containing monofluoroacetate consumed by the animals. This case demonstrates the diagnostic value of using modern chemical instrumentation to detect toxins in gastrointestinal contents from animals intoxicated by poisonous plants.

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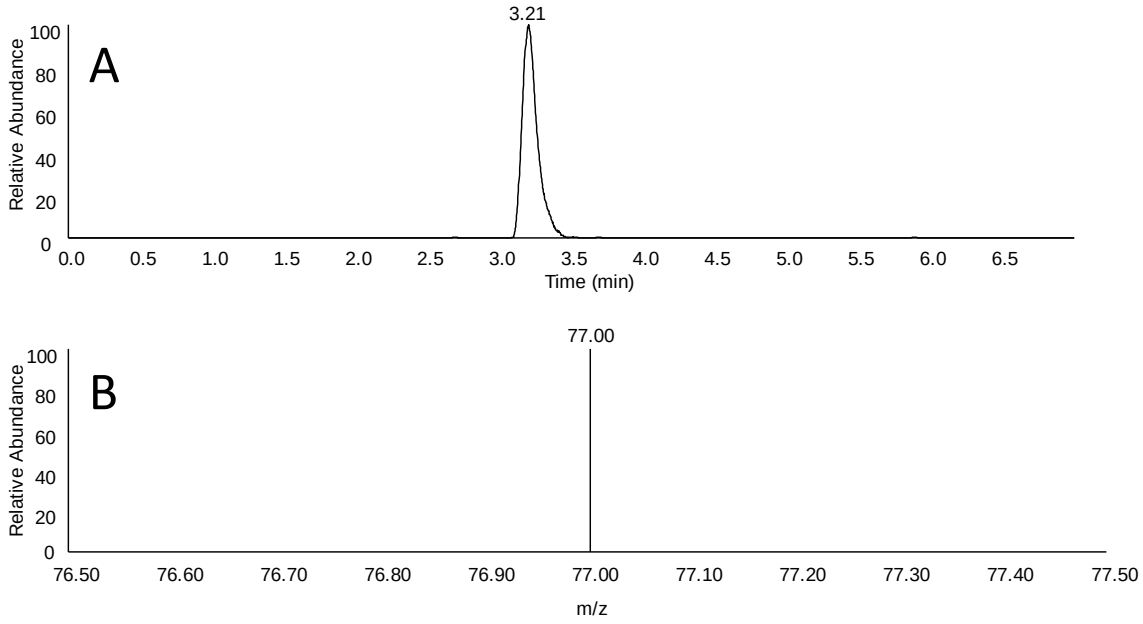

Figure 2. Selected negative ion monitoring HPLC chromatogram at *m/z* 77; of (A) water extract of sheep rumen plant material and (B) the mass spectrum of the peak. *m/z*, mass to charge ratio.

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Poisoning by *Astragalus garbancillo* var*. garbancillo* in Sheep in Northwestern Argentina

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Abstract

Several toxic plants produce lysosomal storage of glycoproteins and mainly nervous clinical disorders. A disease caused by the consumption of *Astragalus garbancillo* var. *garbancillo* in sheep from northwestern Argentina is described here. The affected animals presented weight loss, listlessness, staggering gait, and ataxia. Histopathological studies revealed cellular vacuolation in several organs, mainly in the central nervous system (CNS). The material accumulated in the cells was positive for lectins LCA (*Lens culinaris* agglutinin), sWGA (succinyl–*Triticum vulgaris* agglutinin), and Con A (*Concanavalin* A). Finally, the presence of toxic levels of swainsonine was detected in composite samples of the plant. Poisoning by *Astragalus garbancillo* var. *garbancillo* was characterized as α-mannosidosis of plant origin.

Keywords: Northwestern Argentina, sheep, swainsonine, *Astragalus garbancillo* var. *garbancillo*

Introduction

Glycogenosis caused by the consumption of plants of the *Swainsona* genera in Australia and *Oxytropis* and *Astragalus* in many other parts of the world has been known for years (Huang et al. 2003). However, several species in these taxonomic groups are not toxic and even have potential as good forage resources (Cook et al. 2014).

The genus *Astragalus* is widely distributed and comprises between 2,500 and 3,000 species (Niknam and Lisar 2004), with 110 endemic species being present in South America from Ecuador, Peru, and Bolivia to Chile and Argentina. In Argentina, 70 species occur (Zuloaga et al. 2008) from Jujuy to Tierra del Fuego, mainly in the Andean-Patagonian region (Gómez-Sosa 1979, 1994; Daviña and Gómez-Sosa 1993). According to Gómez Sosa (2004), *Astragalus garbancillo* grows from northern Peru through Bolivia to central-western Argentina at elevations of 1,600-4,500 m. The author identifies two varieties: *A. garbancillo* var. *garbancillo* and *A. garbancillo* var. *mandoni*, which differ in the tip of the flower keel. Only the variety *garbancillo* grows in Argentina (Gómez Sosa 2004). The toxicity in the genus *Astragalus* is due to the presence of

swainsonine, an alkaloid that can inhibit lysosomal α-mannosidase and Golgi α-mannosidase II (Cook et al. 2014).

Williams and Gomez Sosa (1986) determined nitro compounds in 30 species of *Astragalus* in Argentina. Swainsonine has been measured only in *A. pehuenches* (Molyneux & Gómez-Sosa 1991, Robles et al. 2000) and now in *A. garbancillo*. Endophytic fungi have been shown to be responsible for swainsonine production in *Astragalus* and other plants (Cook et. al. 2014). The possible involvement of fungi in both *A. pehuenches* and *A. garbancillo* remains to be studied.

The present work describes locoweed cases in sheep associated with the consumption of *Astragalus garbancillo* var. *garbancillo* (*A. garbancillo*) in northwestern Argentina.

Materials and Methods

The study consisted of four visits to the locality of Pampa Llana, 26°61'S, 66°28'W, 3655 m, San Carlos department, Salta province, Argentina, where nervousness and death in several sheep flocks was described. The area corresponds to the Puna biogeographic region and is characterized by a dry and cold climate with large daily temperature variations reaching absolute minimum values down to –15 ºC (Reboratti 2005). The evaluation of the situation involved visiting five farmers and asking them about the disease. During the interviews, two criollo sheep affected by the condition mentioned above were identified. One of the animals was a 4-year-old ewe with obvious signs of nervousness (animal I), and the other was a 6-year-old ewe showing progressive weight loss and depression (animal II). Both animals were euthanized by administration of an overdose of xylazine or ketamine, and complete post-mortem examination was performed. Tissue samples from CNS, pancreas, heart, liver, and kidney were taken, fixed in 10% buffered formalin, and processed following the common procedure for histological examination. Selected sections of the CNS were subjected to lectin histochemistry using previously described procedures (Driemeier et al. 2000). Finally, suspected plants were collected from different areas of the grazing sites, pressed, and dried for identification at the MCNS Herbarium of the National University of Salta (Holmgren et al. 1990). Part of the plant material was dried to obtain a composite sample. This sample was used to determine swainsonine (SW) using the methods described by Gardner et al. (2001). Results are expressed in % SW (Dry Matter, DM).

Results

Clinical and Epidemiological Findings

All of the farmers stated that between 0.5 and 2% of the flock is affected every year and that the problem is more severe in dry years, with cases usually occurring between August and December. The two affected animals belonged to different flocks that grazed in different areas of the same region. Animal I exhibited weight loss, listlessness, staggering gait, neck and head tremors, ataxia, and difficulty in standing. When animal I attempted to raise its head, the signs increased, and the animal showed the inability to stand, remaining instead in a dog's sitting position. Animal II exhibited gradual weight loss, pale mucous membranes, listlessness, and overall poor condition. Finally, all producers associated the observed disease with the consumption of *A. garbancillo*, indicating that, once affected, the animals leave the flock, walk erratically, and tend to consume the plant compulsively.

Macroscopic Findings

The observed macroscopic changes include lack of subcutaneous and abdominal fat. The animal with nervous signs exhibited mild bruises resulting from the difficulty standing (figure 1).

Figure 1. Adult sheep affected by the disease as shown in the sheep's "dog-sitting" position.

Botanical Identification and Analysis of Swainsonine

All the samples were identified as *A. garbancillo* var. *garbancillo* and recorded as specimen MCNS 12880 at the MCNS Herbarium of the National University of Salta. Swainsonine concentration in the composite samples of leaves and fine stems ground at early vegetative stage was 0.030% (in March 2014) and 0.034% (in December 2014) (figure 2).

Figure 2. *Astragalus garbancillo* var. *garbancillo*.

Histopathological Findings

Histopathological analyses in both animals revealed vacuolation of the neuronal cytoplasm, especially in cerebellum and basal nuclei. In animal II, vacuolation was less evident, and a loss of a large number of Purkinje cells in cerebellar folia was observed. Many of the remaining cells exhibited necrosis with pyknotic and condensed cytoplasm (figure 3). All these results are detailed in table 1. Vacuolation also extended to other tissues, such as the myocardium, liver, kidney, and pancreas. In the pancreas, vacuolation affected the exocrine cells, and islets of Langerhans exhibited atrophy (table 2).

Lectin Histochemistry

Results of lectin histochemistry are shown in table 2. Different labeling patterns were observed between animals. In animal I, the cells exhibited a “foamy” pattern, with multiple coalescent vacuoles in the cytoplasm. In animal II, the affected neurons exhibited a solid and homogenous, condensed labeling pattern. Some of these cells also exhibited a densely granulated cytoplasm (figure 4).

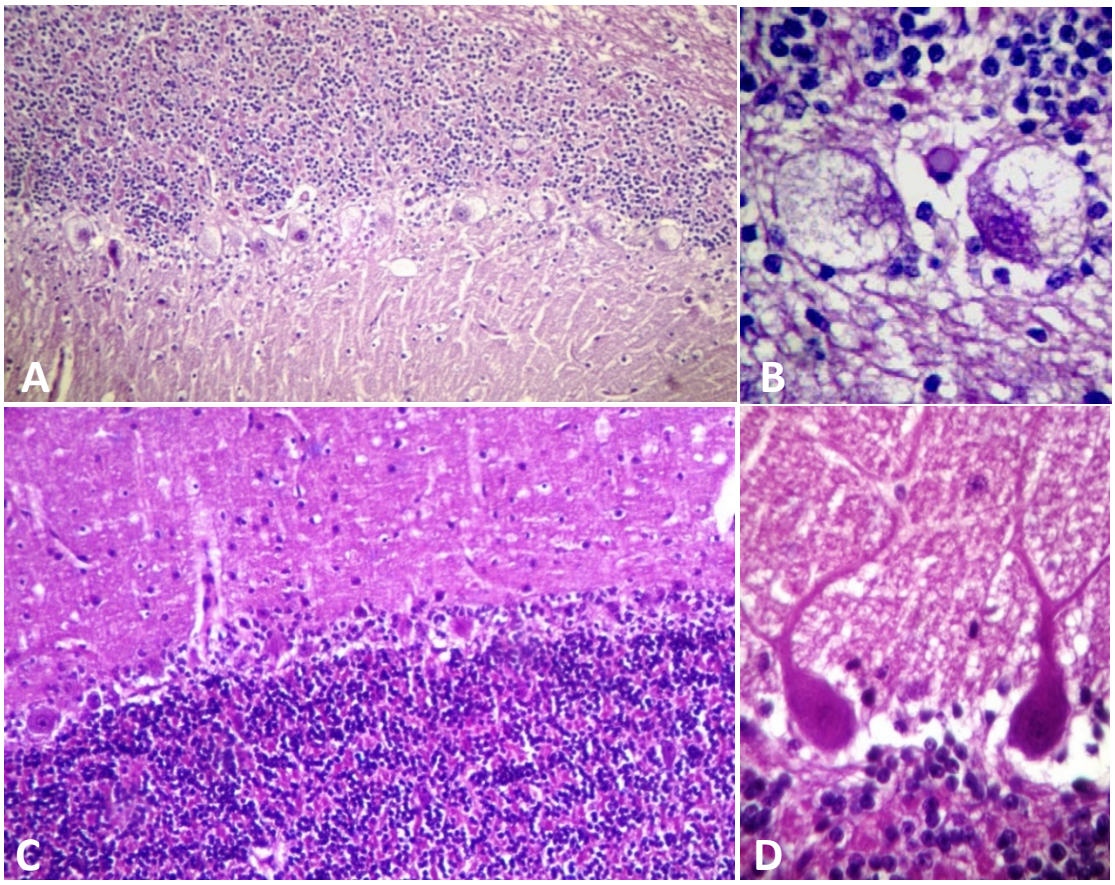

Figure 3. Animal I: (A) Severe vacuolation of the Purkinje cells (H&E 40X). (B) Detail of affected Purkinje cells (H&E 400X). Animal II: (C) Remarkable loss of Purkinje cells in the cerebellar folia. Retraction of the scarce remaining cells (H&E, 40X). (D) Detail of cellular lesions showing cytoplasmic retraction (H&E 400X).

Table 1. Histopathological findings in nervous system

Animal	Samples	Neuronal loss	Vacuolar changes	Gliosis
I	Cerebral cortex	-	++	+
	Basal ganglia	-	++++	+
	Cerebellum	+	++++	-
II	Cerebral cortex	-	+	++
	Basal ganglia	++	+	+++
	Cerebellum	+++	++	+++

- = no / + = minimal + + = mild ++ + = moderate ++++ = severe

Table 2. Lectinhistochemical analysis in nervous system

Animal	Samples	LCA	BS1	WGA	sWGA	Con A
I	Cerebellum	+	-	+	+	+
	Basal ganglia	+	-	+	+	+
II	Cerebellum	+	-	-	-	+
	Basal ganglia	+	-	+	+	+

+ = positive - = negative

Discussion

The clinical and pathological findings, along with the regional history and the concentration of swainsonine found in *A. garbancillo* var. *garbancillo* in the two sampling periods, allow us to attribute the severe clinical cases observed to the consumption of this plant. Swainsonine concentrations were about 0.030% of DM in both sampling periods; levels above 0.001% are considered harmful for livestock (Molyneux et al. 1994). Stegelmeier et al. (1999) indicated that doses of swainsonine equal to or above 0.2 mg/kg/day for at least 21 days can produce irreversible neurological disease.

Sheep appear to be particularly sensitive to the effects of swainsonine, which has been attributed to differences in the toxicokinetics of the alkaloid (Stegelmeier et al. 1995). The observed vacuolation in tissues is a typical characteristic of poisoning by plants containing swainsonine (Jolly and Walkley 1997), and the histological differences found between the two cases studied can be attributed to different stages of disease development. Lesions similar to those found in animal II were mentioned for goats naturally intoxicated with *Ipomoea carnea* (De Balogh et al. 1999), which indicated more chronic stages of the disease as the possible causes for these findings. James and Van Kampen (1971) also mentioned the presence of residual lesions in experimentally poisoned horses and cattle that were euthanized 1 month after the end of plant consumption. Accordingly, the accumulation of sugars in cytoplasm may lead to cellular death (Dorling et al. 1989, Elbein 1989, Takeda et al. 2014), which is in agreement with the high loss of Purkinje cells in the cerebellum observed in animal II.

In agreement with other cases of α-mannosidosis, lectin histochemistry showed positive reactivity with Con-A, WGA, sWGA, and LCA in cytoplasmic vacuoles of Purkinje cells (Stegelmeier et al. 2005). As with histological differences, the different labeling patterns observed may be due to the different development stages of the disease. Lectin labeling with Con A and LCA suggests accumulation of mannose, whereas WGA and sWGA indicate accumulation of N-acetyl-D-glucosamine and sialic acid (Goldstein and Hayes 1978, Goldstein 1980). Thus, the type of sugar accumulated by the cells would vary with stage of disease progress both in the cerebellum and in the basal nuclei.

Other normally affected tissues are the reproductive organs, the nervous system, and the endocrine and immunological system (Elbein et al. 1981, Driemeier et al. 2000, McLain et al. 2004, Stegelmeier et al. 2005, Dantas & Riet-Correa 2007, Ríos et al. 2008, 2015). In this case, pancreatic, hepatic, and renal lesions described agree with those produced by other plant species that induce lysosomal accumulation due to the presence of swainsonine (Van Kampen and James 1972, Driemeier et al. 2000, Dantas and Riet-Correa 2007, Ríos et al. 2008). Cardiac lesions observed seem to

be much less frequent, although they were described in experimentally poisoned rats (Stegelmeier et al.1995). Therefore, the myocardium seems to be more resistant to the action of swainsonine than other tissues. Accordingly, sheep experimentally intoxicated with *Astragalus lentiginosus* did not reveal cardiac lesions 32 days after administration of the plant (Van Kampen and James 1972).

Molecular phylogenetic studies of South American species of *Astragalus* grouped the species in two different clades (Wojciechowski et al. 1999). Scherson et al. (2008) included *A. garbancillo* in a subclade with several species including *Astragalus amatus, A. arnotianus, A. berteroanus, A. cruckshanksii, A. cryptobotrys, A. nivicola,* and *A. uniflorus*. However, none of these species have been reported as being toxic due to the presence of swainsonine. Swainsonine levels found in *A. garbancillo* var. *garbancillo* were similar to those reported in other species (Ralphs et al. 2008). However, the concentration of swainsonine may vary according to the presence of endophytes, the rate of growth, and environmental stress conditions (Ralphs et al. 2008).

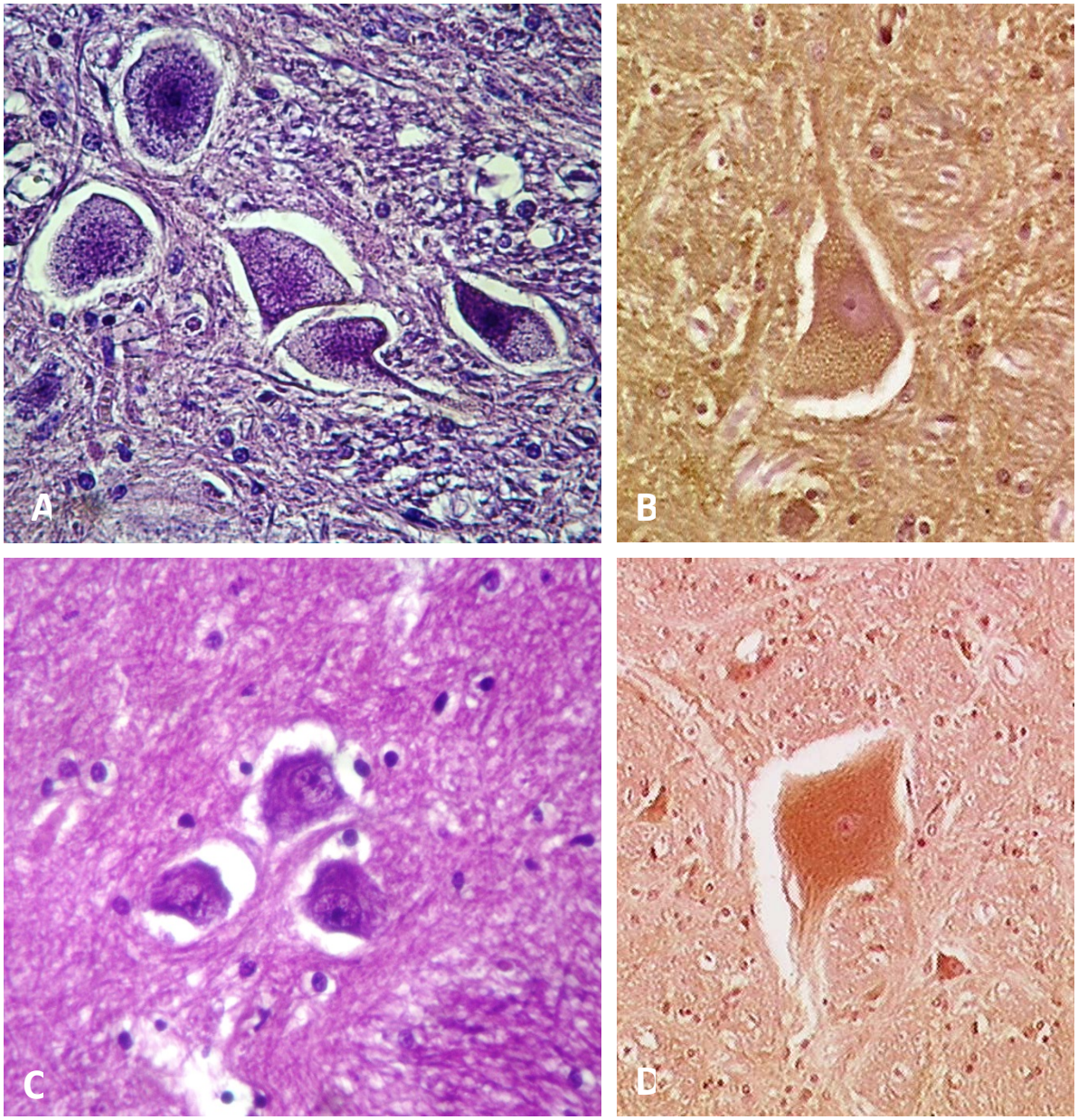

Figure 4. Animal I: (A) Severe neuronal vacuolation at the basal cerebellar nuclei (H&E 40X). (B) Vacuolated perikaryon positive for LCA (400X). Animal II: (C) neurons at the basal cerebellar nuclei showed condensed perikaryon (H&E 40X) and labeled positive with LCA (400X).

Overall, the observed clinical signs, the histopathological lesions of vacuolation, and neuronal loss associated with lectin labeling allow us to conclude that the animals were affected by α-mannosidosis. The abundance of *A. garbancillo* var. *garbancillo* in the grazing areas and the high swainsonine levels detected in the two sampling periods, along with the knowledge of the local farmers, suggest that this plant was the cause of the condition. The variations in the lesion patterns observed, as well as the diversity of the genus in South America, require further studies on this topic.

Acknowledgments

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Lupine Poisoning in Sheep on the USDA-ARS U.S. Sheep Experiment Station (USSES), Dubois, Idaho

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Abstract

The USDA-ARS United States Sheep Experiment Station (USSES), located north of Dubois, ID, manages approximately 13,759 hectares of rangeland in Idaho for range research, sheep breeding and nutrition research, and rangeland management. Over 210 sheep deaths occurred in 3 separate summer/fall grazing periods from 2004 to 2011. Death losses occurred in ram lambs (86; 2004), mature rams (12; 2010), and ewe lambs (112; 2011). Between 1978 and 1981, multiple losses were also reported; however, data representing exact numbers were not recorded. All reported death losses occurred on the Humphrey Ranch, and information recorded from 1985 to the present identified four specific pastures as problematic. Lupine samples were randomly collected throughout the headquarters and the Humphrey Ranch. Specimens were collected for chemical analysis and taxonomic identification. Five lupine species were identified: *Lupinus caudatus*, *L. leucophyllus*, *L. polyphyllus*, *L. sericeus*, and *L. argenteus*. Each lupine species contained a distinct chemical profile composed of quinolizidine and/or piperidine alkaloids.

Keywords: alkaloid, lupine, *Lupinus*, piperidine, poisoning, quinolizidine, rangelands, sheep

Introduction

The *Lupinus* genus contains more than 500 taxa of annual, perennial, or soft woody shrub-like species worldwide (Wink et al. 1995): 200-300 species in North and South America, 150 species in the Intermountain West of the United States (Cronquist et al. 1989), 95 species in California (Riggins and Sholars 1993), and 12 species in Europe and Africa (Wink et al. 1995).

Range lupines are found in a variety of habitats at all elevations from lowland deserts to the alpine crests (Kingsbury 1964). Most lupines in the continental United States grow in States and provinces west of the Cordilleras including the Rocky Mountains and Sierra Nevada extending northward to Alaska and southward into Chile in South America (Wink et al. 1995). Lupines are found eastward through the Great Plains to the Atlantic coast but consist of only relatively few annual and perennial species.

Stockmen in the western United States began to recognize the inherent danger of lupines late in the 1800s when large livestock losses, especially in sheep, were reported in Montana and other western States (Chesnut and Wilcox 1901). Most poisonings occurred in late summer or early fall or when sheep were fed “native” hay containing lupine in the winter. These losses were often sporadic and continued throughout the late 1800s and early 1900s (Sampson and Malmsten 1942, Stoddart and Smith 1955). Use of lupine hay for winter feed was greatly scrutinized following the winter of 1898-1899 when thousands of sheep died from consuming lupine hay

containing large numbers of seed pods (Chesnut and Wilcox 1901). In one flock alone, 3,600 of 7,000 sheep died from eating lupine hay. Most ranchers that season lost over 50 percent of their flocks to lupine hay poisoning. Retrospective analysis determined that during the summer of 1898, most lupine hay was harvested early, between July 1 and July 20, and it was reported that the lupine had "formed an unusual quantity of seeds." While alkaloids had not yet been described, it was clear that the seed pods were "rich in the poison" (Chesnut and Wilcox 1901). Due to research and changing grazing practices, large sheep losses from lupines are rare today. However, lupines continue to cause periodic sheep losses and large and even catastrophic losses to cattle producers from lupine-induced crooked calf syndrome in the western United States and Canada (Panter et al. 1997, 2013; Gay et al. 2007).

Lupines are very diverse, ranging from cultivated low-alkaloid species (sweet lupines) used for human and animal food to toxic wild species (bitter lupines) that have multiple alkaloids and are responsible for toxicoses and teratogenesis in livestock. Taxonomically, lupine species are difficult to classify because of extensive hybridization, lack of morphological uniformity, and absence of genetic barriers to interbreeding (Cronquist et al. 1989). Chemical profiles can support or contradict taxonomic identification but are essential for establishing risk of poisoning to livestock. For example, Cook and colleagues described seven chemotypes of *Lupinus sulphureus* alone and used alkaloid profiling (chemotaxonomy) to differentiate between three other lupine species and *L. sulphureus* (Cook et al. 2009, 2011). Alkaloid profiles in some lupines vary considerably within and between species, making risk assessment using taxonomy alone unreliable (Carey and Wink 1994, Wink and Carey 1994, Lee et al. 2007, Cook et al. 2009).

The objectives of this report were to (1) identify lupine species and associated sheep losses on the USSES, (2) determine taxonomic identification and chemical profiles for lupine species, and (3) provide basic management recommendations to prevent future sheep losses from lupines.

Materials and Methods

History and Site Description

The USDA-ARS United States Sheep Experiment Station (USSES) is located north of Dubois, ID (figure 1), and consists of approximately 13,759 hectares of rangeland in Idaho for range research, sheep breeding and nutrition research, and rangeland management. On two of the USSES properties, the headquarters (11,303 hectares) and the adjoining Humphrey Ranch (1,052 hectares), *Lupinus* spp. are abundant. Over 210 sheep deaths occurred in 3 separate summer/fall grazing periods from 2004 to 2011. Death losses occurred in ram lambs (86; 2004), mature rams (12; 2010), and ewe lambs (112; 2011). Between 1978 and 1981, multiple losses were also reported; however, data representing exact numbers were not recorded. All reported death losses occurred on the Humphrey Ranch, and information recorded from 1985 to the present identified four specific pastures as problematic. Lupine samples were collected throughout the headquarters and the Humphrey Ranch. Specimens were collected for chemical analysis and taxonomic identification. Five lupine species were identified: *Lupinus caudatus*, *L. leucophyllus*, *L. polyphyllus*, *L. sericeus*, and *L. argenteus*; each contained a distinct alkaloid profile represented by multiple quinolizidine alkaloids and/or piperidine alkaloids.

Plant Collections

Lupine samples were collected randomly throughout the USSES headquarters and the Humphrey Ranch (figure 1) from June through August of 2009-2012 when plants were in full flower and early seed stage. Whole aboveground plant parts, including vegetative and reproductive parts, were collected at several locations for taxonomic evaluation and chemical analysis. Paired specimens were collected, one assigned for taxonomic evaluation and the other for chemical analysis. The taxonomic specimen was pressed, mounted, and submitted to the S.L. Welsh Herbarium (BRY; S. Welsh) at Brigham Young University, Provo, UT, for morphological comparison and taxonomic identification. Once classified, the specimen was assigned a voucher number and permanently filed in the Poisonous Plant Research Laboratory (PPRL) Herbarium, Logan, UT. Subsequently, a small amount of leaf material was removed from the voucher specimen for chemical analysis at the PPRL, and the chemical profile was then compared with the paired sample chemically analyzed for verification.

Alkaloid Analysis

The plant material sampled from the pressed specimens and plant material from field samples were ground to pass through a 2 mm screen, stored at room temperature, and submitted for chemical

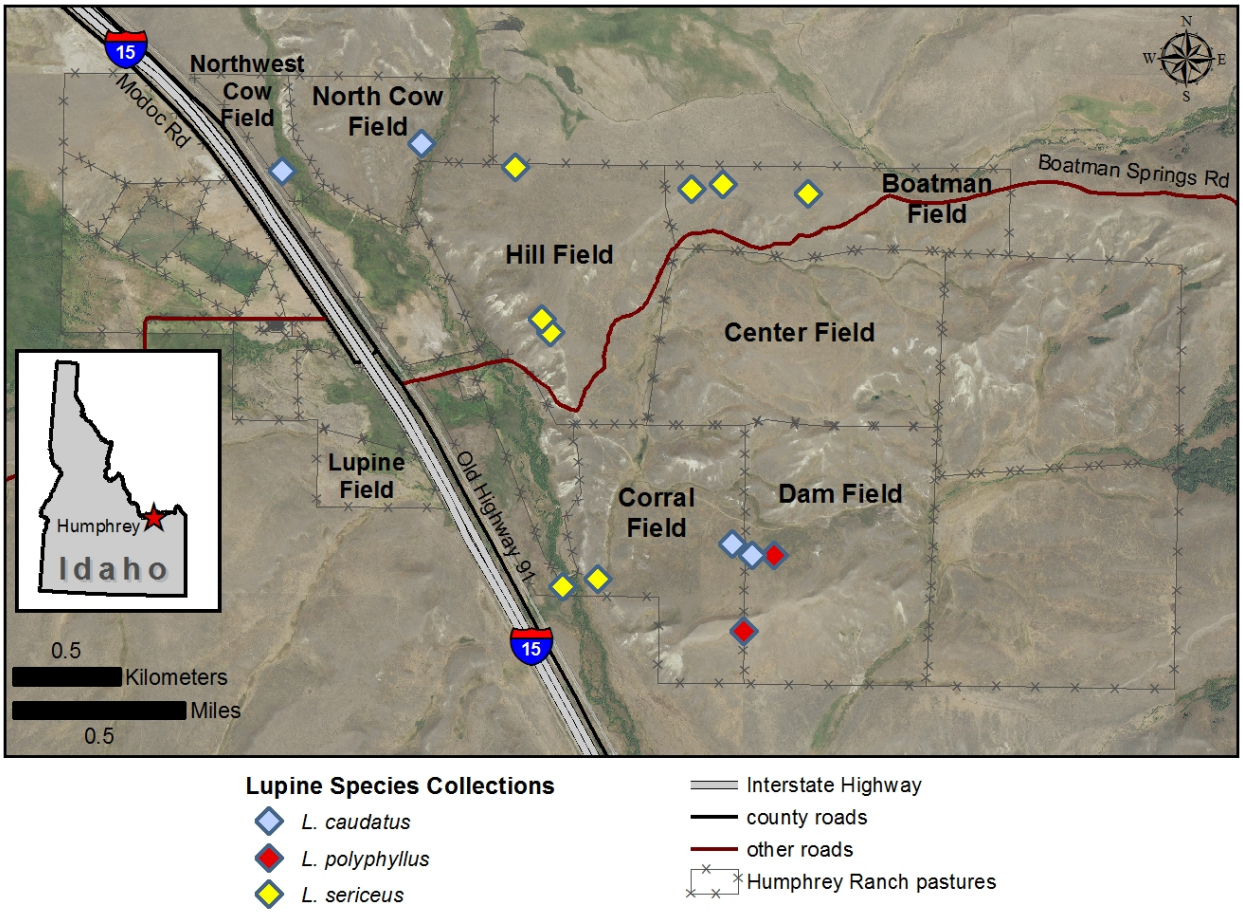

Figure 1. Map of the USDA-ARS U.S. Sheep Experiment Station Humphrey Ranch, with lupine sample collection sites, which are identified by a diamond symbol. Pastures where past lupine poisoning events with sheep have occurred (see table 1) are Dam Field (2004), Center Field (2010), Corral Field (1985 and 2011), and Lupine Field (estimated 1978-1981).

extraction and analysis (Lee et al. 2007). Briefly, a measured quantity (50 mg herbarium sample, 100 mg field collection) of the ground plant material was weighed into a 16 mL screw-top glass test tube. The plant material was extracted by mechanical rotation using the Rugged Rotator (Glas Col, LLC) with a mixture of 1 *N* HCl (4.0 mL) and $CHCl_3$ (4.0 mL) for 15 min. The samples were centrifuged (5 min) and the aqueous layer removed. An additional 2.0 mL of 1 *N* HCl was added to the test tube containing plant material and $CHCl_3$ and extracted again by mechanical rotation (15 min), centrifuged, and the aqueous layer removed. The aqueous portions were combined into a clean 16 mL screw-top glass test tube. The pH of the aqueous layer was adjusted to 9.0-9.5 with concentrated NH_4OH. The basic solution was extracted twice with $CHCl_3$, first with 4.0 mL and then with 2.0 mL. The $CHCl_3$ solutions were combined and filtered through anhydrous Na_2SO_4 into a clean 16 mL screw-top glass test tube, and the solvent evaporated under N_2 at 60 °C. The alkaloid fraction extracted was reconstituted in 2 mL (herbarium samples) or 4 mL of methanol (field collections) containing 1.3 µg/mL caffeine (internal standard). A portion (~1 mL) was transferred to 1.5 mL gas chromatography (GC) autosampler vials for GC/mass spectrometry (MS) analysis.

GC/MS analysis was performed (Lee et al. 2007). In brief, representative samples (2 µL) of each plant sample were analyzed by GC/MS using a Polaris Q mass spectrometer and Trace GC Ultra gas chromatograph (Thermo Electron Corp.) equipped with a split/splitless injector and a DB-5MS (30 m x 0.25 mm; J&W Scientific) column. Injection port temperature was 250 °C and operated in the splitless mode. Split vent flow rate was 50 mL/min and purged after 0.80 min. Oven temperature was 100 °C for 1 min, 100-200 °C at 40 °C/min, 200-275 °C at 5 °C/min; and held at 275 °C for 1.5 min. Electron impact ionization (EI) at 70 eV was used with an ion source temperature of 200 °C. The detector scanned the mass range *m/z* 50-650.

Alkaloid identification was performed (Lee et al. 2007). Four individual alkaloids were identified from commercially obtained standards [sparteine and lupanine and authenticated (MS, NMR) samples of ammodendrine and anagyrine from the PPRL alkaloid collection]. The remaining alkaloids were determined from correlation of measured retention times to retention indices (RI) calculated by linear extrapolation from RI values generated from known standards and assigned RI numbers from the literature and their electron ionization (EI) and chemical ionization (CI) mass spectra (Wink et al. 1995). Alkaloids were also determined by correlation of measured relative retention times (RR_t) to lupanine and EI mass spectra to those reported in the literature (Kinghorn and Balandrin 1984).

Results and Discussion

Five lupine species were identified on the two properties of the USSES: *L. argenteus* (PPRL Accession #3678 and #3801), *L. caudatus* (PPRL Accession #4499 and #4503), *L. leucophyllus* (PPRL Accession #3800, #4497, and #4498), *L. polyphyllus* (PPRL Accession #3802, #4494, and #4496), and *L. sericeus* (PPRL Accession #4500, #4501, #4502, and #4504). Each lupine contained a diagnostic alkaloid profile composed of quinolizidine and piperidine alkaloids (figures 2-6). The alkaloid profile of *L. sericeus* was quite simple and contained one major quinolizidine alkaloid, lamprolobine. The alkaloid profiles of *L. argenteus, L. caudatus, L. leucophyllus,* and *L. polyphyllus* were more complex and contained three or more major quinolizidine alkaloids. All five *Lupinus* species could pose a toxic risk to grazing livestock.

More than 150 quinolizidine alkaloids and several piperidine alkaloids have been identified from the *Lupinus* genera (Keeler and Gross 1980, Schmeller et al. 1994). Lupine alkaloids are toxic; however, toxicity varies depending on structural features of individual alkaloids, and toxicity in animals depends on alkaloid profiles, total alkaloid concentration, and rate of plant ingestion. Typically, plant alkaloid content is elevated during early phenological stages, decreasing through the flower stage, and increases in pods and seeds through translocation (Keeler et al. 1976, Lee et al. 2006). The clinical signs of lupine poisoning begin with nervousness, depression, grinding of the teeth, frothing round the mouth, relaxation of the nictitating membrane of the eye, frequent urination and defecation, and lethargy (Panter et al. 1999). These signs progress to muscular weakness and fasciculations, ataxia, collapse, sternal recumbency leading to lateral recumbency, respiratory failure,

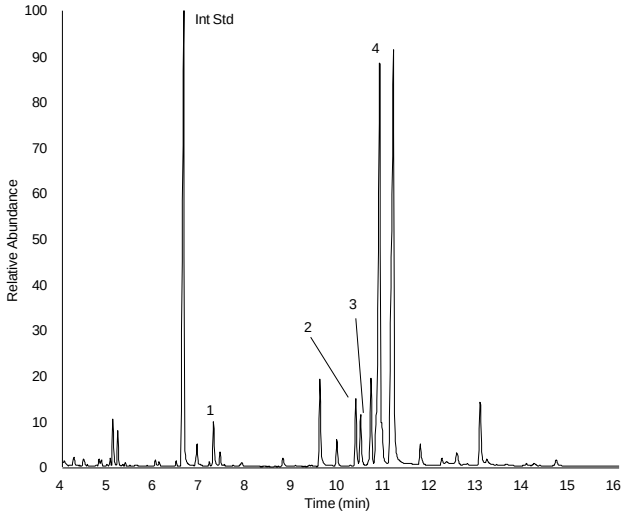

Figure 2. Gas chromatogram of the alkaloid profile representative of *L. argenteus*. Peaks annotated on the chromatogram: internal standard, caffeine (Int Std); ammodendrine (1); 5,6 dehydrolupanine isomer (2); 5,6 dehydrolupanine (3); and lamprolobine (4). All other peaks are unknown.

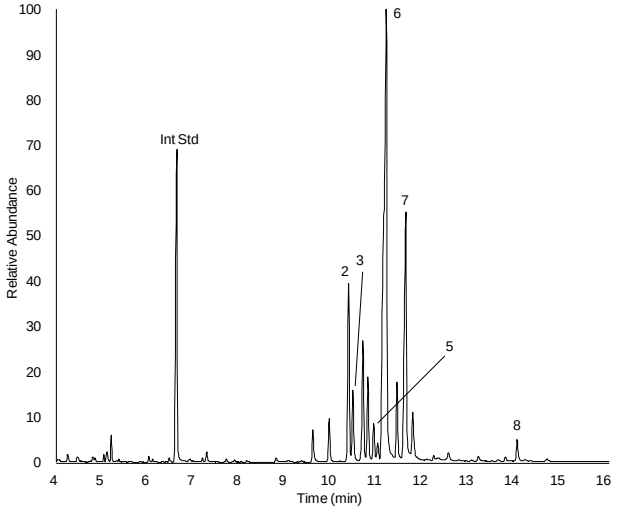

Figure 3. Gas chromatogram of the alkaloid profile representative of *L. caudatus*. Peaks annotated on the chromatogram: internal standard, caffeine (Int Std); 5,6 dehydrolupanine isomer (2); 5,6 dehydrolupanine (3); lupanine (5); (2*R*)-hydroxyaphyllidine (6); (2*S*)-hydroxyaphyllidine (6); (2*R*,9*R*)-dihydroxyaphyllidine (7); (2*S*,9*R*)-dihydroxyaphyllidine (7); and anagyrine (8). All other peaks are unknown.

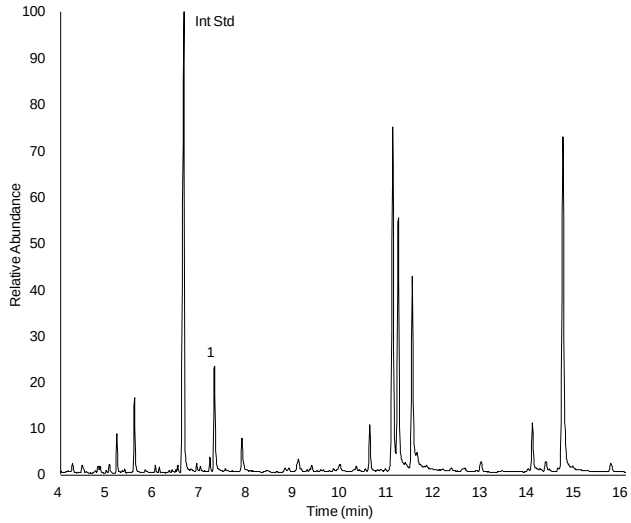

Figure 4. Gas chromatogram of the alkaloid profile representative of *L. leucophyllus*. Peaks annotated on the chromatogram: internal standard, caffeine (Int Std) and ammodendrine (1). All other peaks are unknown.

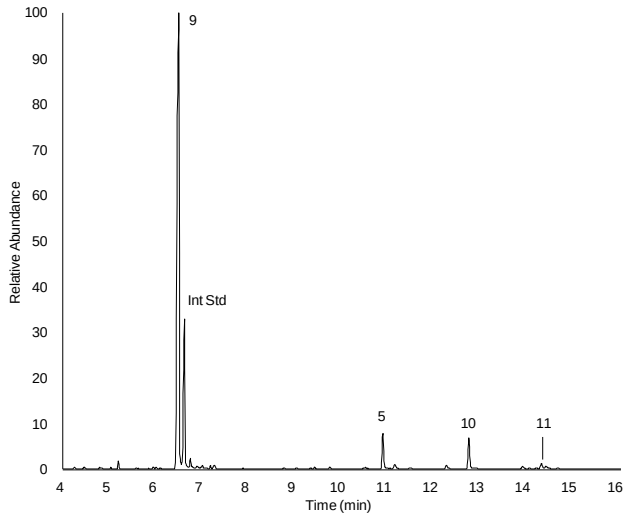

Figure 5. Gas chromatogram of the alkaloid profile representative of *L. polyphyllus*. Peaks annotated on the chromatogram: internal standard, caffeine (Int Std); lupanine (5); spartiene (9); 13β-hydroxylupanine (10); and 13α-hydroxylupanine (11). All other peaks are unknown.

and death. Clinical signs may appear as early as 1 hour after ingestion and progressively get worse over the course of 24 to 48 hours even if further ingestion does not occur. Generally, if death does not occur within this timeframe, the animal recovers completely. The clinical signs of poisoning are the same in sheep and cattle and are due to the effects of quinolizidine and piperidine alkaloids on the peripheral and central nervous systems.

Three of the *Lupinus* spp. could cause "crooked calf syndrome" (CCS) cattle, although no cattle

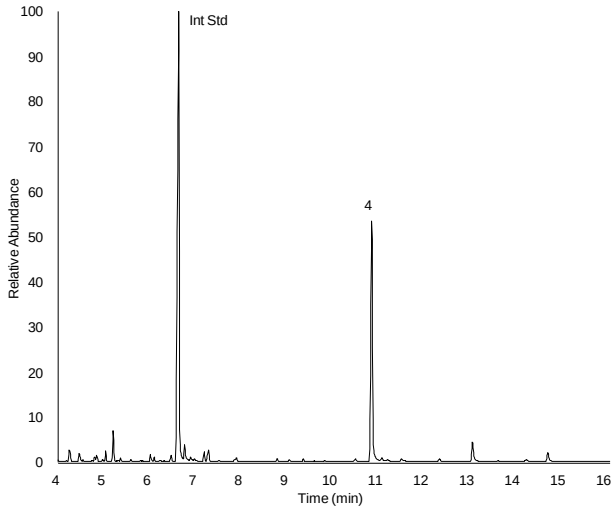

Figure 6. Gas chromatogram of the alkaloid profile representative of *L. sericeus.* Peaks annotated on the chromatogram: internal standard, caffeine (Int Std) and lamprolobine (4). All other peaks are unknown.

graze on USSES. Crooked calf syndrome is a condition in which calves are born with a variety of skeletal deformities such as arthrogryposis, scoliosis, kyphosis, torticollis, and cleft palate (Shupe et al. 1967a, 1967b, 1968). The principal time of insult was identified as the 40-70th days of gestation and may extend to as late as day 100. Anagyrine (Keeler et al. 1976) and some piperidine alkaloids (Keeler and Panter 1989, Panter et al. 1998) were shown to reduce fetal movement during this critical period of gestation (Panter et al. 1990, Panter and Keeler 1992), causing the spine and limbs to develop in contracted or misaligned positions and occasional cleft palate. Cattle losses in several western U.S. States due to lupine-induced CCS are still prevalent today (Lee et al. 2009, Panter et al. 2013). *L. caudatus* on the USSES contained the teratogenic alkaloid anagyrine while *L. leucophyllus* and *L. argenteus* contained the suspected teratogen ammodendrine. If pregnant cattle grazed these pastures during days 40-100 of gestation, there would be a significant risk of CCS because of the presence and concentrations of anagyrine and ammodendrine found in these species (Panter et al. 1999). While death losses occur more frequently in sheep than in cattle, cattle deaths are occasionally reported (Panter et al. 2001). However, the greater impact in cattle is CCS, which continues to cause large economic losses to cattle producers in western United States and Canada (Panter et al. 2013).

Documented sheep death losses on the USSES from lupine have occurred on the Humphrey Ranch (table 1). Based on this limited survey, three of the

Table 1. Historical account of lupine-induced sheep deaths at the USDA-ARS U.S. Sheep Experiment Station Humphrey Ranch research location

Year	Month	Animal type	Death toll	Pasture[1]	Lupine prevalence[2]
2011	September	ewe lambs	112	Corral Field	Concentrated under sagebrush
2010	July	mature rams	12	Center Field	Concentrated along ridge tops
2004	September	ram lambs	86	Dam Field	Concentrated around a secondary water source
1985	September	ram lambs	multiple	Corral Field	Concentrated under sagebrush
1978-1981[3]	Fall	sheep	multiple	Lupine Field	Unknown

[1]Refer to figure 1 for pasture location.
[2]Specific location characteristics of high-density lupine infestations that were grazed by sheep.
[3]Specific dates were not recorded but were determined by historical notes and personal communications with U.S. Sheep Experiment Station employees.

five lupine species identified on the USSES were found on the Humphrey Ranch pastures (*L. caudatus*, *L. polyphyllus*, and *L. sericeus*) while four lupine species were found on the headquarter pastures (*L. argenteus*, *L. caudatus*, *L. polyphyllus*, and *L. leucophyllus*). *Lupinus polyphyllus* and *L. caudatus* were both collected from the Corral Field where 112 sheep died in 2011; however, *L. sericeus* was the most prevalent species collected at the Humphrey Ranch. No plant samples were collected from the Lupine Field (figure 1) as this pasture had undergone recent rangeland improvements following multiple herbicide applications in past years to mitigate the historic sheep losses that occurred from 1978-1981. In contrast, *L. leucophyllus* and *L. polyphyllus* were the most prevalent species collected at headquarters.

Although *Lupinus* spp. can be found on all USSES properties, historic records indicate that under the current management practices, the greatest risk for sheep loss is on the Humphrey Ranch. *Lupinus* spp. were well distributed across the Humphrey Ranch in 1981. To fully evaluate lupine populations, long-term vegetation monitoring transects should be established and surveys conducted with taxonomic identification and chemical verification.

In the mid- to late 1980s, various attempts (herbicide application, pasture renovation, etc.) were used to control lupine infestations on the Lupine Field, and this resulted in a substantial reduction of lupine on the property west of Interstate Highway I-15 (figure 1). Subsequent to this, all the sheep losses occurred on pastures east of I-15 (figure 1). Some mitigation efforts (herbicide) were attempted east of I-15 in the late 1980s and early 1990s. Although the success of these efforts was not quantified, one could speculate that the lack of poisoning incidences from 1985 to 2004 could suggest some degree of lupine control.

Beginning in 2004 and repeated in 2010 and 2011, significant lupine poisoning events were documented (table 1) at the Humphrey Ranch. Most losses occurred within a 400 acre area, which is divided among three adjoining pastures: Center Field, Corral Field, and Dam Field (figure 1). In the spring of 2004, a pre-grazing inspection was conducted prior to moving sheep into the pastures on the Humphrey Ranch location. According to USSES records, visible *Lupinus* spp. were in the vegetative stage with very few flowers present, and it was determined that risk of poisoning was low or at least presumed acceptable for grazing. However, poisoning occurred, and 86 ram lambs died. Three lupine species were identified in these areas: *L. caudatus*, *L. polyphyllus*, and *L. sericeus*. Immediately after the poisoning event, isolated, dense populations of lupine plants with pods were found in the areas where sheep had grazed. These isolated “patches” of lupine were reported to be remote and somewhat obstructed from view of the road, such as along ridge tops, concentrated under sagebrush, or along secondary watering sources that appear in wet years (table 1).

In this report, we identified five lupine species and documented five distinct alkaloid profiles within these species. This report suggests that *L. sericeus*, *L. polyphyllus*, and/or *L. caudatus* were most likely responsible for the sheep losses that have occurred on the USSES rangelands. However, more investigation and research needs to be done to determine if one of these species is primarily

responsible for the majority of the losses. With the knowledge that lupine was responsible for the sheep deaths, appropriate mitigation efforts should be implemented to reduce sheep losses due to lupine grazing on the USSES pastures in the future.

Conclusions and Management Recommendations

These results provide important information to rangeland and sheep managers on the USSES to mitigate further sheep losses and to implement future lupine control measures. There are multiple management approaches that will reduce or eliminate sheep losses including one or more of the following recommendations: (1) Evaluate rangelands and identify poisonous plants and elucidate potential risk. This report provides a good example for livestock producers to evaluate risk from poisonous plants. (2) Targeted herbicide control of concentrated patches of lupines or broad generalized control. Recommended herbicides include 2,4-D (2 lb ae/acre), 2,4-D plus dicamba (1 + 0.5 lb ae/acre), or triclopyr (0.5 to 1.5 lb ae/acre) (Ralphs et al. 1991). Spray actively growing plants after they are 5 inches high but before bloom (Panter et al. 2011). Retreatment may be necessary every 4 to 5 years because viable seed reserves in the soil persist for many years. (3) Utilize high-risk pastures early in the growing season when other lush forage is available. Generally, livestock will avoid poisonous plants when adequate good-quality forage is available. Also, early grazing may reduce flowering success of lupines and inhibit pod development, further reducing risk of poisoning. (4) Graze high-risk pastures late in the growing season after lupine pods have shattered. Once lupine pods have shattered, alkaloid levels are very low, and risk of poisoning is substantially reduced. (5) Avoid driving animals through lupine patches or unloading hungry animals near poisonous plant populations. (6) Do not bed sheep near patches of poisonous plants, and do not place salt or water near populations of poisonous plants. Avoid creating situations where sheep travel through poisonous plants to get access to salt, water, or supplements. This report can serve as a template for investigating other cases of sheep or cattle losses associated with lupines or other poisonous plants.

While some lupine species are readily grazed by livestock and contain substantial nutritional qualities, the risk of poisoning can only be determined by alkaloid analysis and risk assessment (Panter et al. 2001, 2013). It is recommended that lupine plants (preferably while in flower) be submitted to the Poisonous Plant Research Laboratory, Logan, UT, for taxonomic identification and chemical analysis before animals are turned out for grazing.

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