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The effect of low larkspur (*Delphinium* spp.) co-administration on the acute toxicity of death camas (*Zigadenus* spp.) in sheep

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ABSTRACT

In most cases where livestock are poisoned by plants in a range setting, there is more than one potential poisonous plant in the same area. Two poisonous plants that are often found growing simultaneously in the same location are death camas (*Zigadenus* spp.) and low larkspur (*Delphinium* spp.). Sheep are known to be susceptible to death camas poisoning while they are thought to be resistant to larkspur. The objective of this study was to determine if co-administration of low larkspur would exacerbate the toxicity of death camas in sheep. A dose finding study was performed to find a dose of death camas that caused minimal clinical signs of poisoning. Sheep were observed for clinical signs of poisoning as well as changes in heart rate and muscle fatigue. Sheep dosed with 1.14 g of death camas per kg BW showed slight frothing and lethargy, whereas sheep dosed with death camas and low larkspur showed slightly more noticeable clinical signs of poisoning. Sheep dosed with only low larkspur, at 7.8 g/kg BW, showed no signs of poisoning. Although we observed a qualitative difference in clinical signs of intoxication in sheep co-treated with death camas and low larkspur we did not detect any quantitative differences in heart rate, exercise-induced muscle fatigue, or differences in serum zygacine kinetics. Consequently, the results from this study suggest that low larkspur does not affect the toxicity of death camas in sheep. The results from this study increase knowledge and understanding regarding the acute toxicity of death camas and low larkspur in sheep. As combined intoxications are most likely common, this information will be useful in further developing management recommendations for ranchers and in designing additional experiments to study the toxicity of death camas to other livestock species.

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1. Introduction

In most cases where livestock are poisoned by plants in a range setting, there are multiple poisonous plants in the area. Two poisonous plants that are often found growing simultaneously in the same location are death camas (*Zigadenus* spp.) and low larkspur (*Delphinium* spp.). Both plants emerge early in the spring and exhibit similar phenological growth stages. Poisonings generally occur during the spring when these plants are abundant, while other forage species have little growth. Poisonings may also occur on overgrazed ranges where better quality forage has been depleted, or when management errors result in hungry animals being moved into death camas/larkspur-infested areas (Panter et al., 1987). Livestock losses to death camas have been reported in numerous species with

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the largest losses generally occurring in sheep (Kingsbury, 1964). Sheep are primarily affected because of their tendency to select forbs, particularly in the early spring when there is little other plant growth.

Low larkspur poisonings cause large economic losses to cattle producers in the western United States and Canada (Pfister et al., 2003). The primary result of larkspur intoxication is neuromuscular paralysis. However, larkspurs are five times more toxic to cattle than sheep (Olsen, 1978). Sheep might be less susceptible to larkspur because the binding affinity of alkaloids to nAChRs is lower in sheep than in cattle (Stegelmeier et al., 1998). Consequently low larkspur is not regarded as a high risk poisonous plant for sheep. However, there is potential that consumption of low larkspur by sheep may exacerbate the acute toxic effects of plants such as death camas. It has been demonstrated that co-treatment of mice with the primary toxins from death camas and low larkspur has an additive effect (Welch et al., 2011). Therefore, the objective of this study was to determine if the co-administration of low larkspur will exacerbate the acute toxicity of death camas in sheep.

2. Materials and methods

2.1. Plant material

Death camas (*Zigadenus paniculatus*) was collected in the late pod stage near Logan, Utah (N lat 41°46.098′ W long 111°46.688′, at an elevation of approximately 1550 m, PPRL collection 81-9). Low larkspur (*Delphinium andersonii*) was collected in the flowering stage near Picabo, ID (N lat 43°14.813′ W long 114°13.300′, at an elevation of approximately 1475 m, PPRL collection 11-2). Each collection of plant material was air-dried, and ground to pass through a 2.4 mm screen using a Gehl Mix-All model 55 (Gehl Company, West Bend, WI, USA). After processing, the ground plant material was stored in plastic bags away from direct light at ambient temperature in an enclosed shed until use, with plant alkaloid analyses performed immediately prior to use.

2.2. Plant alkaloid analyses

Samples of dry ground death camas were extracted following a procedure previously described for larkspur alkaloids (Gardner et al., 1997). The evaporated extracts were dissolved in 1.0 mL of chloroform and a 0.010 mL aliquot was taken and dissolved in 0.990 mL 50% methanol (0.1% formic acid) in an autosampler vial. Analyses were completed using an LCQ Advantage Max (Thermo Scientific) mass spectrometer coupled with a Surveyor autosampler plus and MS pump plus (Thermo Scientific) used in-line with a Gemini NX column (100 × 2 mm) with a guard column of equivalent phase. A binary solvent gradient using 0.1% formic acid (solvent A) and acetonitrile (solvent B), at a flow rate of 0.300 mL/min and the following gradient mixture with time: 10% B (0–1 min); 10–50% B (1–10 min); 50% B (10–11 min); 50-10% B (11–12 min); 10% B (12–18 min). The flow from the column was connected to an electrospray ion source. The mass spectrometer was set to a full scan range of 100–800 *m/z* and peak areas were measured from reconstructed ion chromatograms (*m/z* 536.2 selected for zygacine) and were analyzed using purified zygacine (0.5 mg/mL) standard curve at a concentration range of 10, 5, 2.5, 1.25, 0.625 μg/mL. Injection volume was 5 μL.

The low larkspur collection was analyzed for *N*-(methylsuccinimido) anthranoyllycoctonine (MSAL) alkaloid content using HPLC-mass spectrometery, as previously described (Gardner and Pfister, 2009). The major MSAL alkaloids detected were 16-deacetylgeyerline (16-DAG; 19.50 min), methyllycaconitine (MLA; 20.82 min), geyerline (21.03 min), 14-deacetylnudicauline (14-DAN; approximately 21.25 min), nudicauline (21.91 min), and 14-acetylbearline (24.57 min) (Fig. 1B). There were also small amounts of 7, 8-methylenedioxylycoctonine (MDL) alkaloids detected (14.03, 14.91 and 16.72 min).

2.3. Animals

Cross-bred western white-faced sheep weighing 44 ± 6 kg were used. Animals were housed in a large outdoor pen and maintained on alfalfa for at least one week prior to dosing. The afternoon prior to dosing, the sheep were moved to an enclosed barn with ad libitum access to water but not feed. Plant material was administered to sheep in a water slurry via oral gavage. Two hours after dosing, animals were provided access to alfalfa hay. Approximately 30 h after dosing the sheep were returned to the outdoor pens. All procedures were conducted under veterinary supervision and were approved by the Utah State University Institutional Animal Care and Use Committee, #2112.

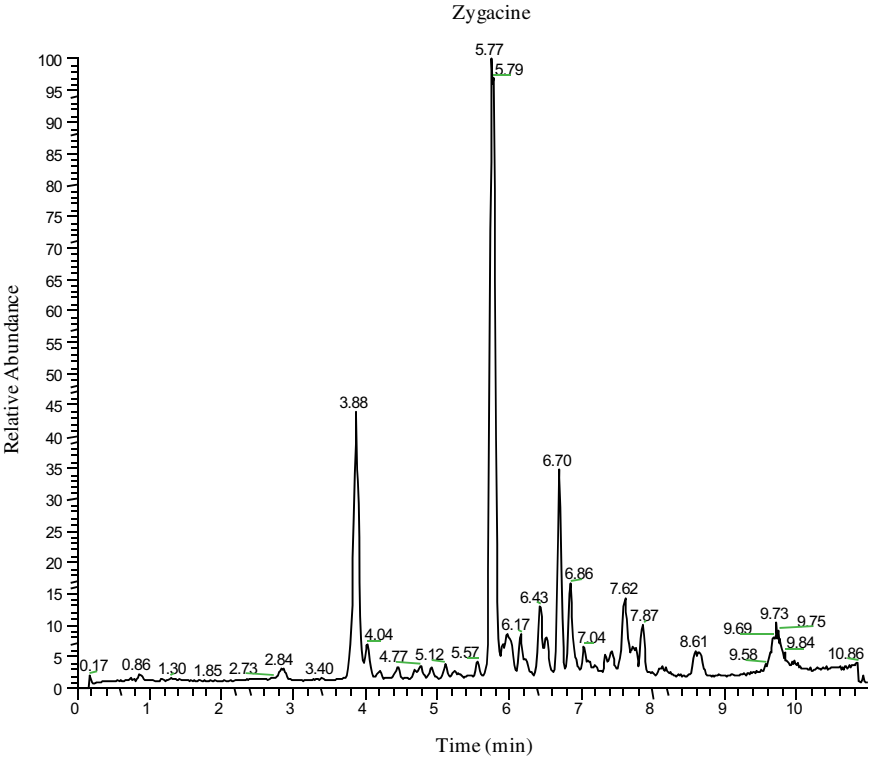
2.4. Dose-response analysis

Sheep were dosed with death camas via oral gavage at 8, 10, 12, 14, 16, and 18 mg zygacine/kg BW. Sheep were observed for clinical signs of intoxication for 24 h after dosing. A minimum of two sheep were treated at each dose.

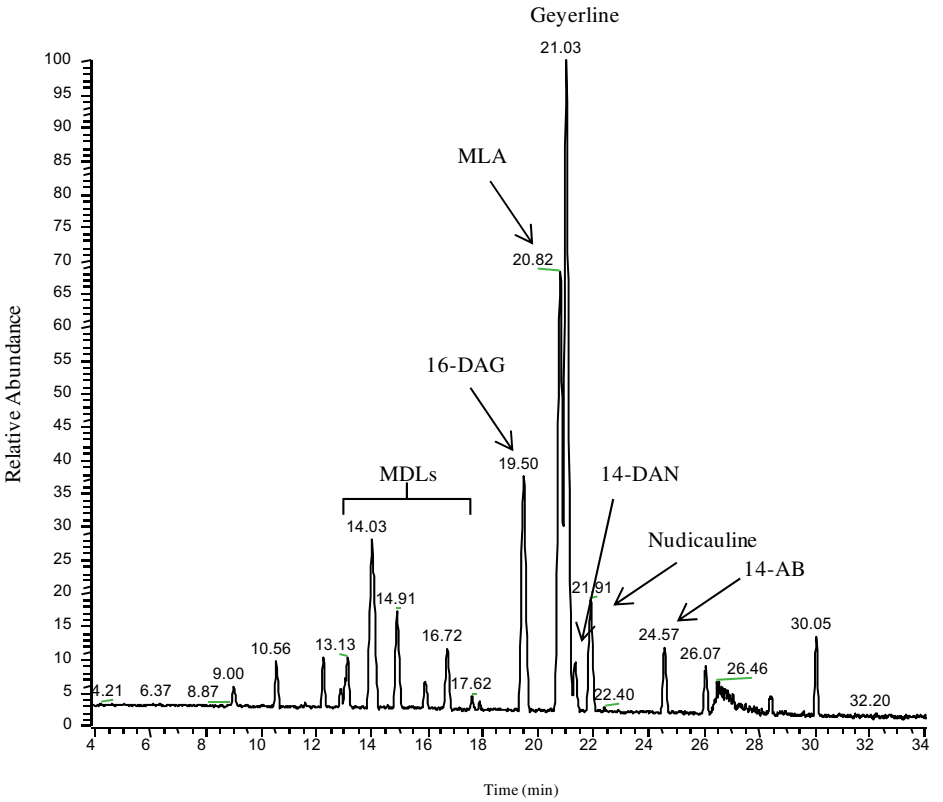
2.5. Heart rate analyses

Heart rate was monitored as outlined previously (Green et al., 2009b). Briefly, data were recorded using an AD Instruments Powerlab, and signals were amplified with an ML132 amplifier (AD Instruments Inc. Colorado Springs, CO, USA). Heart rate was monitored using 3M Red Dot model 2670 repositionable monitoring electrodes (3M Corporation, St. Paul, MN, USA) secured in place with a gel-based formulation of cyanoacrylate adhesive (Henkel Consumer Adhesive, Inc. Avon, OH, USA). The leads were placed as described by (Chen et al., 2002) with the positive electrode placed on the right scapula and the negative electrode on the sternum adjacent to the heart. A ground electrode was attached on the back. The heart rate signal was amplified with a gain range of ±500 μV. The heart rate signal was filtered with a mains filter, 60 Hz notch filter, 120 Hz low-pass; 0.1 Hz high-pass filter and digital band-pass filter with a high cut-off frequency of 45 Hz and a low cut-off frequency of 0.1 Hz. The cyclic measurements feature of ADI Chart software package was used to calculate

A) Death Camas



B) Low Larkspur



heart rate in beats per min (BPM). Fifteen min periods of heart rate were measured prior to dosing and then 4, 8, and 24 h after dosing. The heart rate in each animal was allowed to stabilize before analysis (typically 5 min). After stabilization a 5-min period of heart rate was sampled.

2.6. Toxicokinetic analyses

For toxicokinetic studies, 8 sheep weighing 50 ± 8 kg were randomly divided into 2 groups of 4 sheep each. One group received death camas alone, while the other group received a combination of death camas and low larkspur. Sheep were dosed with death camas at 1.14 g/kg BW, which corresponded to 8.0 mg zygacine/kg BW. Low larkspur was dosed at 2.28 g/kg BW, which corresponded to 4.6 mg MSAL alkaloids/kg BW. Blood was collected at 16 time points after dosing (0, 0.5, 1, 2, 4, 6, 9, 12, 24, 29, 34, 48, 58, 72, 82 and 96 h). The serum fraction of the blood samples was separated and stored at −20 °C until analysis.

2.7. Serum analyses

Matrix matched standards were prepared for methyllycaconitine, a common MSAL alkaloid, and zygacine as follows: A stock solution of methyllycaconitine was prepared at 1 mg/mL in ethanol and then 0.020 mL diluted with 0.980 mL ethanol to provide a 20 ppm solution. A 0.050 mL aliquot was added to 1.950 mL of blank sheep sera and serially diluted with sheep sera to give matrix standards at 500, 250, 125, 62, 31, 16 and 8 ng/mL methyllycaconitine. A 0.020 mL aliquot of zygacine stock solution (0.50 mg/mL in ethanol) was diluted in 0.980 mL of ethanol to give a 10 μg/mL solution from which 0.020 mL was diluted in 1.980 mL ethanol to give a 100 ng/mL solution. A 0.200 mL aliquot was then diluted with 1.800 mL of blank sheep sera and serially diluted to give zygacine standards at 5, 2.5, 1.25, 0.62, 0.31 and 0.16 ng/mL.

Sheep serum samples were thawed, vortexed and then centrifuged for 5 min. For the sera samples, and the matrix standards, a 0.500 mL aliquot was taken and placed in a 1.5 mL eppendorf tube. An equal volume of acetonitrile (0.500 mL) containing 250 ng/mL reserpine was added to each sample. Samples were vortexed for 10–15 s and then centrifuged at 16,000 × g for 10 min. A 0.75 mL aliquot was then removed to a 1.5 mL autosample vial for analysis.

Both methyllycaconitine and zygacine were analyzed by HPLC–tandem mass spectrometry. However, because of their different level of concentration in the sera they were analyzed under slightly different conditions. For the analysis of methyllycaconitine an LCQ Advantage Max (Thermo Scientific) mass spectrometer coupled with a Surveyor autosampler plus and MS pump plus (Thermo Scientific) was used in-line with a Betasil C18 column (100 × 2.1 mm) with a guard column of equivalent phase. The column was eluted with a binary solvent gradient using 0.1% formic acid (solvent A) and acetonitrile (solvent B), at a flow rate of 0.300 mL/min and the following gradient mixture with time: 15% B (0–1 min); 15–75% B (1–8 min); 75% B (8–10 min); 75-15% B (10–11 min); 15% B (11–16 min). The flow from the column was connected to an electrospray ion source. The mass spectrometer was set to scan selected MS/MS experiments during the following time segments: (5.25–6.80 min) MLA parent ion *m*/*z* 683.3 with CID fragmentation power of 35; and (6.8–10 min) reserpine parent ion *m*/*z* 609.2 with CID fragmentation power of 33. Reconstructed ion chromatograms used the following selected ions for methyllycaconitine (619.3, 651.3 and 665.3) and for reserpine (397.1 and 448.2). Reserpine was used as an internal injection standard and calibration and quantitation was completed using peak areas from reconstructed ion chromatograms for methyllycaconitine versus reserpine.

For the analysis of zygacine an LTQ Velos Pro (Thermo Fisher Scientific) mass spectrometer coupled with a Agilent 1100 binary pump and Agilent autosampler was used in-line with a Betasil C18 column (100 × 2.1 mm) with a guard column of equivalent phase and eluted with a binary solvent gradient using 20 mM ammonium acetate (solvent A) and acetonitrile (solvent B), at a flow rate of 0.300 mL/min and the following gradient mixture with time: 5–50% B (0–8 min); 50–100% B (8–14 min) with flow increased to 0.400 mL/min; 100% B (14–18 min); 100–5% B (18–19 min) with flow reduced to 0.300 mL/min; 5% B (19–25 min). The flow from the column was connected to a heated electrospray ion source. The mass spectrometer was set to scan selected MS/MS experiments during selected time segments: (0–11 min) zygacine parent ion *m*/*z* 536.2 with CID fragmentation power of 25; and (11–18 min) reserpine parent ion *m*/*z* 609.2 with CID fragmentation power of 28. Reconstructed ion chromatograms used the following selected ions for zygacine (500.3 and 518.3) and for reserpine (397.1 and 448.2). Reserpine was used as an internal injection standard and calibration and quantitation was completed using peak areas from reconstructed ion chromatograms for zygacine versus reserpine.

2.8. Exercise

Sheep were dosed orally with dried, ground plant material. Fatigue and weakness were assessed by exercising the sheep 4, 8, and 24 h after dosing. Sheep were exercised in groups of four by walking them back and forth in a 100 m alley at a rate of approximately 3 km/h for 10 min. If an animal became fatigued to the point that it could not maintain a 3 km/h pace, it was allowed to fall behind the others. The number of animals that could, and could not, walk for 10 min for each group was noted.

Fig. 1. Reconstructed base peak ion chromatograms from HPLC-mass spectrometry analysis of death camas and low larkspur. Zygacine was the major alkaloid in the death camas plant material. In the low larkspur plant material, the major *N*-(methylsuccinimido) anthranoyllycoctonine (MSAL) alkaloids detected were 16-deacetylgeyerline (16-DAG; 19.50 min), methyllycaconitine (MLA; 20.82 min), geyerline (21.03 min), 14-deacetylnudicauline (14-DAN; approximately 21.25 min), nudicauline (21.91 min), and 14-acetylbearline (24.57 min). There were also small amounts of 7, 8-methylenedioxylycoctonine (MDL) alkaloids detected (14.03, 14.91 and 16.72 min).

2.9. Data analysis and statistics

Statistical comparisons of serum alkaloid profiles were performed using ANOVA with a Bonferroni posthoc test of significance between individual groups using SigmaStat for Windows (version 3.1, SPSS Inc., Richmond, CA). Statistical comparisons between 2 groups were performed using a 2-tailed, unpaired Students *T*-test. The number of sheep that collapsed due to exercise-induced muscle fatigue was analyzed by comparing two groups within a time point using a 2 × 2 contingency table with a Fisher's Exact Test. The alkaloid concentrations were plotted using SigmaPlot for Windows (version 9.0, SPSS Inc., Richmond, CA). Kinetic profiles were analyzed using standard pharmacokinetic software (PK Solutions 2.0 for Non compartmental Pharmacokinetic Data Analysis, Summit Research Services, Montrose, CO; 1998). A curve-stripping procedure was used to determine the basic pharmacokinetic parameters of half-life and rate for the elimination phase from blood of the zygacine concentration curve. The following parameters were determined: absorption rate, elimination rate, maximum alkaloid concentration (C_{max}), time to maximum serum concentration (T_{max}), and area under the curve ($AUC_{0\text{-}\infty}$). A trapezoidal method was used to determine the AUC of a concentration vs. time graph.

3. Results

The death camas used for this study was foothill death camas (*Zigadenus paniculatus*), which contained 0.7% zygacine (on a dry weight basis). The low larkspur used for this study was (*D. andersonii*), which contained 0.2% MSAL alkaloids (on a dry weight basis), which included the alkaloids 14-deacetylnudicauline (14-DAN), 16-deacetylgeyerline (16-DAG), methyllycaconitine (MLA), nudicauline, 14-acetylbearline (14-AB) and geyerline (Fig. 1).

A dose–response experiment was conducted to determine a dose of death camas that would cause minimal signs of intoxication. The amount of death camas administered was based upon the concentration of zygacine. Consequently, sheep were dosed at 8, 10, 12, 14, 16, and 18 mg/kg zygacine (Table 1). Sheep dosed with 8 mg/kg zygacine had minimal clinical signs of poisoning, with slight frothing in some of the sheep and some animals appearing slightly lethargic. However, by 8 h post-dosing, all animals visually appeared normal. In general, as the dose of zygacine increased so did the severity of clinical signs, with increased amounts of frothing, lethargy, dyspnea, and vomiting. The time to recover also correlated with dose, such that animals receiving larger doses of death camas took longer to recover.

Death camas is known to cause cardiovascular deficiencies. Consequently, the effects of death camas treatment on heart rate and EKG were assessed. Six groups of 4–6 sheep each were compared: death camas alone (1.14 g/kg death camas or 8.0 mg/kg zygacine), death camas plus an equal amount of low larkspur plant material (1.14 g/kg death camas and 1.14 g/kg low larkspur), death camas plus 2× low larkspur (1.14 g/kg death camas and 2.28 g/kg low larkspur), 2× death camas (2.28 g/kg death camas), 2× death camas plus an equal amount of low larkspur plant material (2.28 g/kg death camas and 2.28 g/kg low larkspur), and low larkspur alone (7.79 g/kg low larkspur). Heart rate in sheep was evaluated for 15 min immediately before dosing and again at 4, 8, and 24 h post-dosing (Table 2). There was no difference in heart rate between groups ($P = 0.798$). There was a difference in time ($P = 0.011$) with the animals, in general, having the highest heart rate at 8 h post dosing. There was no group × time interaction ($P = 0.902$). Due to the extremely large variation among animals, there were no consistent, obvious changes in EKG over time or between groups (data not shown). Even though the co-treatment of low larkspur with death camas had no additional effect on heart rate, the animals that were co-treated with both plants had more severe clinical signs of poisoning, including labored breathing, than the animals treated with death camas alone.

The alkaloids in low larkspur inhibit normal neuromuscular function causing muscle weakness and collapse in poisoned animals. This muscle weakness can be exacerbated by physically stressing the animals. Consequently, we evaluated the effect of low larkspur and death camas co-exposure on exercise-induced muscle weakness and fatigue. For this experiment, four groups of four sheep each were used, including control (1.14 g/kg alfalfa), death camas alone (1.14 g/kg death camas), death camas plus 2× low larkspur (1.14 g/kg death camas and 2.28 g/kg low larkspur), and low larkspur alone (2.28 g/kg low larkspur). The sheep were exercised by walking them back and forth across a 100 m alley at approximately 3 km/h for 10 min at 4, 8, and 24 h post-dosing. The number of sheep in each group that were physically unable to maintain this pace was noted (Table 3). Statistically there was no difference (*P* values ranged from 1.0 to 0.43) in the number of sheep that collapsed in each group at each time point. However, there was a trend for a greater effect in the sheep receiving both death camas and low larkspur, especially at the 8 and 24 h time points. Again, animals treated with low larkspur and death camas showed more severe clinical signs than

Table 1
Dose-response evaluation of clinical signs of toxicity in sheep fed death camas.

Group	*n*	Plant (g/kg)	Zygacine (mg/kg)	Time to recover (h)	Clinical signs
8 mg/kg	6	1.14	8.0	8	Slight frothing, slightly lethargic
10 mg/kg	2	1.43	10.0	10	Frothing, slight vomiting, lethargic
12 mg/kg	2	1.71	12.0	24	Frothing, vomiting, lethargic, dyspnea, grinding teeth
14 mg/kg	2	2.00	14.0	24	Frothing, vomiting, weakness, dyspnea
16 mg/kg	6	2.28	16.0	24	Frothing, vomiting, weakness, dyspnea
18 mg/kg	2	2.57	18.0	>24	Frothing, vomiting, weakness, dyspnea, grinding teeth

Table 2
The effect of death camas and low larkspur co-treatment on heart rate in sheep.

Treatment	*n*	Death camas (g/kg)	Low larkspur (g/kg)	Zygacine (mg/kg)	MSAL (mg/kg)	Heart rate (BPM)			
						$t = 0$ h	$t = 4$ h	$t = 8$ h	$t = 24$ h
DC	6	1.14	0.0	8.0	0.0	78 ± 5	76 ± 8	90 ± 15	88 ± 5
DC + 1× LL	4	1.14	1.14	8.0	2.3	73 ± 4	75 ± 6	70 ± 2	79 ± 4
DC + 2× LL	4	1.14	2.28	8.0	4.6	69 ± 5	69 ± 2	86 ± 22	82 ± 4
2× DC	6	2.28	0.0	16.0	0.0	82 ± 7	75 ± 12	91 ± 13	77 ± 9
2× DC + LL	4	2.28	2.28	16.0	4.6	79 ± 15	79 ± 10	89 ± 6	100 ± 11
LL	4	0.0	7.79	0.0	15.6	68 ± 3	67 ± 2	91 ± 6	83 ± 6

Note: Heart rate was assessed for 15 min immediately prior to dosing and then at 4, 8, and 24 h after dosing. Sheep were dosed orally with death camas (DC) alone, death camas and low larkspur (LL), or low larkspur alone. Data represent the mean ± SEM of a 5 min selection of the raw trace calculated as beats per minute (BPM) using the cyclic measurements function of the Chart software.

the sheep treated with death camas alone. An important note was that in no instance, in any of the experiments performed in this study, did sheep receiving low larkspur alone demonstrate any clinical signs of poisoning, which supports previous findings that sheep are very resistant to larkspur poisoning.

A toxicokinetic analysis was performed to determine if co-exposure of larkspur and death camas alkaloids alters the kinetic profile of zygacine. There was no difference ($P = 0.077$) in the serum zygacine concentration between sheep dosed with death camas alone versus sheep dosed with death camas and low larkspur (Fig. 2). There was a time effect ($P < 0.001$), but there was no group × time effect ($P = 0.946$). Additionally, there were no differences in the any of the kinetic parameters for zygacine between sheep dosed with death camas alone versus sheep dosed with death camas and low larkspur (Fig. 2). One interesting note was the small quantity of serum zygacine detected ($C_{max} = 1.6$ ng/mL and AUC = 7.1 ng*h/mL) and the short serum half life of zygacine ($E_{1/2} = 1.4$ h). The serum zygacine concentration was below the limit of detection by 30 h post-dosing.

4. Discussion

In most rangeland settings there are multiple poisonous plants growing in the same area (Burrows and Tyrl, 2001; Kingsbury, 1964). Consequently, animals are potentially exposed to multiple poisonous plants containing multiple toxins. For many of the poisonous plants, basic toxicological information is available such as the LD_{50}, the mechanism of action, and the clinical signs associated with their toxicoses (Burrows et al., 2004; Burrows and Tyrl, 2001; Panter et al., 2012). However, very rarely is there information available on the effects of animals consuming combinations of these poisonous plants. Thus, questions remain as to whether consumption of multiple poisonous plants by an animal could have an additive, synergistic, or antagonistic effect. There is potential that the toxicosis elicited by one toxin could potentiate the toxicity of another toxin such that a sub-lethal dose of each toxin, when combined, could produce a lethal result (Davis and Hatoum, 1980; Hellmann et al., 2010; Huff et al., 1988; Welch et al., 2008). In this regard, our previous research demonstrated that co-administration of MLA and zygacine to mice had an additive effect (Welch et al., 2011).

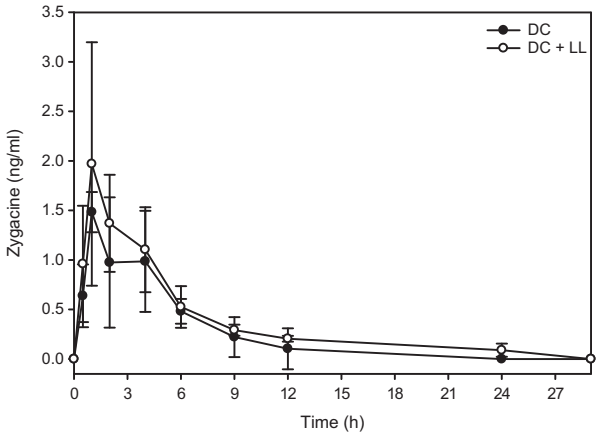
In the mouse model, the LD_{50} value for a 1:1 mixture of MLA and zygacine (2.9 mg/kg) was different from the LD_{50} value of zygacine alone (2.0 mg/kg; $P < 0.001$) and MLA alone (4.5 mg/kg; $P < 0.001$) (Welch et al., 2011). The i.v. LD_{50} value of the mixture was almost half way between the individual values of each compound. However, in essence, the LD_{50} value for the mixture corresponds to a value of 1.45 mg/kg for each alkaloid, which was significantly lower that the LD_{50} for each individual compound ($P < 0.001$). Consequently, these results suggested that the toxicity is additive when combining these two toxins, at least for an acute i.v. toxicity study in mice. However, it is not known how a ruminant dosed orally would respond. Therefore in this study, sheep were dosed orally to compare individual and combined effects of death camas and low larkspur.

The objective of this study was to determine if the co-administration of low larkspur would exacerbate the toxicity of death camas in sheep. Death camas and low larkspur are commonly found in the same geographical locations and growing at the same time (Burrows and Tyrl, 2001). Thus, foraging animals could potentially consume both plants. In fact, we have repeatedly seen evidence that livestock have consumed both plants (personal observations). Additionally, both zygacine and MLA have been identified in the blood and gastrointestinal (G.I.) contents from poisoned animals (personal observations). Therefore, it is important to compare individual and combined effects of death camas and low larkspur in ruminants and determine if the co-administration of low larkspur would exacerbate the toxicity of death camas in sheep.

Table 3
The effect of death camas and low larkspur co-treatment on exercise-induced muscle weakness in sheep.

Group	4 h		8 h		24 h	
	Yes	No	Yes	No	Yes	No
CNT	0	4	0	4	0	4
DC	1	3	1	3	0	4
DC + LL	0	4	2	2	1	3
LL	0	4	0	4	0	4

Note: Muscle weakness was assessed by exercising the sheep by walking them for 10 min up and down a 100 m alley 4, 8, and 24 h after dosing. Sheep were dosed orally with death camas (DC) alone, death camas and low larkspur (LL), or low larkspur alone. Data represent the number of sheep that collapsed due to muscle weakness (yes), or did not collapse (no), at each time point. Statistical comparisons were performed using Fisher's exact test, using a 2 × 2 contingency table; none of the comparisons of treated and control animals were significantly different ($P > 0.05$).



Group	A rate (1/hr)		E rate (1/hr)		Cmax (ng/ml)		Tmax (hr)		AUC (ng*hr/ml)	
	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD
DC	1.0	0.7	0.5	0.3	1.6	0.3	1.3	0.5	7.1	2.6
DC + LL	1.1	0.4	0.3	0.2	2.1	1.2	0.9	0.3	10.7	1.3
P value	0.776		0.235		0.456		0.245		0.062	

Fig. 2. Comparison of the kinetic profile of zygacine in serum from sheep fed death camas alone versus death camas and low larkspur. Data represent the serum concentration of zygacine from sheep dosed orally with 1.14 g/kg death camas (DC), which corresponded to 8 mg/kg zygacine. The group that received death camas and low larkspur (LL) was dosed simultaneously with 1.14 g/kg of both death camas and low larkspur. Results represent the mean ± SD of the concentration of zygacine in serum for 4 sheep at each time point. The kinetic parameters of absorption rate, elimination rate, maximum concentration (C_{max}), time to maximum concentration (T_{max}), and area under the curve (AUC) are also shown. A statistical comparison of the serum alkaloid concentrations was performed using a two-way ANOVA with a Bonferroni post-hoc analysis. Statistical comparisons of each kinetic parameter were performed using Student's T test, with corresponding P value provided. There were no significant differences ($P < 0.05$) observed between the two treatments for any of the kinetic parameters.

Much is known about the toxicity of larkspur, with the identity and toxicity of the majority of the alkaloids characterized (Green et al., 2009a; Pfister et al., 1999). The i.v. LD_{50} for MLA (one of the major toxic alkaloids in larkspur plants) in mice is approximately 4.5 mg/kg (Panter et al., 2002; Pfister et al., 1999; Welch et al., 2008, 2009). The mechanism of action is via the competitive inhibition of nicotinic acetylcholine receptors (nAChR) causing muscle weakness and death due to respiratory failure (Benn and Jacyno, 1983; Dobelis et al., 1999). Less information is known regarding the toxic alkaloids in death camas. For example, not all of the alkaloids in death camas have been indentified, nor have their toxicities been characterized (Welch et al., 2011). The toxic alkaloids in death camas are steroidal alkaloids of the same class as those in *Veratrum* species (Burrows and Tyrl, 2001; Kupchan, 1959), which are suggested to alter sodium transport in the cardiac nerves (Burrows et al., 2004). The principle alkaloid in most death camas populations is zygacine (Welch et al., 2011). The i.v. LD_{50} for zygacine in mice is approximately 2.0 mg/kg and the oral LD_{50} is approximately 130 mg/kg (Welch et al., 2011). However, research has demonstrated that although zygacine is the major alkaloid in *Z. paniculatus*, additional alkaloids in death camas contribute to its toxicity (Welch et al., 2011). Thus, further studies are needed to identify and characterize the other toxic alkaloids in death camas. The clinical signs of animals poisoned by death camas include ataxia, muscular weakness, trembling, incoordination, discharge of frothy saliva from the mouth and nose, vomition, dyspnea, collapse and death (Panter et al., 1987). Based on the clinical signs observed, the toxic alkaloids in death camas likely affect more than just the sodium channels in the cardiac nerves. However, there is a possibility that the inhibition of nAChRs by larkspur alkaloids could exacerbate the toxicosis caused by death camas alkaloids.

In this study, we found that co-administration of low larkspur and death camas plant material to sheep had a rather minor effect compared to the obvious additive effect observed in the mice studies (Welch et al., 2011). We did not observe any statistically significant differences or changes in heart rate (Table 2), exercise-induced fatigue (Table 3), or serum zygacine kinetics (Fig. 2) between animals treated with death camas alone versus animals treated with death camas and low larkspur. However, we did notice that sheep treated with low larkspur and death camas did appear to have slightly more severe clinical signs of intoxication compared to sheep treated with death camas alone, although this was a subjective observation. The discrepancy between the results of this study with sheep, versus the result in mice could be attributed to the fact that sheep are non-responsive to the toxic effects of low larkspur and thus co-treatment has no further effect simply because larkspur has no effect on sheep. This hypothesis is supported by the fact that the sheep dosed with low larkspur alone did not show any indication of intoxication (Tables 2 and 3) including any signs of toxicity. Conversely, mice are susceptible to the toxic effects of both death camas and low larkspur and thus co-treatment had an additive effect (Welch et al., 2011). Consequently, similar experiments need to be performed in a different livestock species, such as cattle, that are susceptible to both the toxic effects of both plants.

Interesting comparisons can be made between the kinetics of zygacine in mice versus sheep. For the serum kinetics, we presume that elimination from the blood results in elimination from the body and not a redistribution within tissues. The kinetic parameter area under the curve (AUC) provides a general indication as to the bioavailability of a compound, summarily depicting the absorption, distribution and elimination of the compound (Shargel and Yu, 1993). The AUC for zygacine in mice dosed i.v. with purified zygacine was approximately 5550 ng mL^{-1}*min (Welch et al., 2011) whereas in this study the AUC for zygacine in sheep dosed orally with ground plant material was approximately 7 ng mL^{-1}*min. Interestingly the AUC for zygacine was much greater in mice compared to sheep. However, the mice were dosed i.v. with purified zygacine (1.0 mg/kg zygacine), thus the entire dose was immediately bioavailable in mice whereas in the sheep the zygacine had to be absorbed as they were dosed orally with ground plant material (8.0 mg/kg zygacine). Thus, in the sheep a much different profile would be expected due to the slow process of absorption of the alkaloids from the gastrointestinal tract. The different route of exposure could also result in

lack of absorption or metabolism of zygacine by rumen microflora, both of which could explain the lower AUC in sheep.

The rate of elimination is valuable in both i.v. and orally dosed animals, as it dictates how long the toxin will remain in the body (Shargel and Yu, 1993; Shen, 2008). In mice dosed i.v. with purified zygacine, we found the half-life of elimination of zygacine from blood to be approximately 13 min versus 79 min in sheep dosed orally with ground plant material, respectively. An elimination half-life of 13 min in mice indicates that 91 min after i.v. exposure over 99.2% of the zygacine is eliminated from the blood. However, we observed that mice dosed with zygacine demonstrated clinical signs of poisoning beyond 91 min (Welch et al., 2011). There are a number of possible explanations for this discrepancy. One explanation is that the toxic alkaloids in death camas bind to a receptor such that residual amounts of alkaloid, below the limit of detection in blood, would maintain the mice in a state of hypersensitivity. Another possible explanation is that the active compound that binds to the receptor is a metabolite of zygacine and consequently not accounted for in the kinetic analyses of the parent compound. Conversely, in sheep dosed with death camas plant material, an elimination half-life of 79 min was calculated, indicating that it takes 9 h to eliminate 99% of the zygacine. Consistent with this finding, sheep dosed with 8 mg/kg zygacine had no clinical signs of intoxication by 8 h post-dosing. These kinetic parameters are useful in evaluating relative risk and to enhance current management recommendations for animals grazing in death camas infested rangelands.

In summary, the results from this study demonstrate that low larkspur co-treatment has no effect on the toxicity of death camas in sheep. Treatment of sheep with death camas caused clear signs of cardiovascular deficiencies and muscle fatigue. However, co-treatment with low larkspur did not exacerbate those deficiencies. The results from this study also confirmed previous findings that sheep are refractory to the acute toxicity of larkspur. The results from this study provide an increased knowledge and understanding regarding the acute toxicity of death camas in sheep, one of the more susceptible livestock species. This information will be useful in further developing livestock management recommendations for ranchers and in designing additional experiments to study the toxicity of death camas in other livestock species.

Ethical statement

No ethical issue.

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Conflict of interest statement

There is no conflict of interest for this work.

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