IDENTIFICATION AND CULTIVATION OF METHYLLYCACONITINE DEGRADERS FROM WILD RUMINANTS TO PROTECT AGAINST LARKSPUR POISONING IN RANGE CATTLE

by

Savannah Gray Grace

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Animal and Range Sciences

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 2021

DEDICATION

To Connor, my sweet husband, thank you for your continued patience and unwavering support. I love you more than you will ever know.

ACKNOWLEDGEMENTS

Words cannot begin to describe the amount of gratitude I hold for the various faculty, students, and family who have aided in my journey through this exciting but sometimes wild experience. I would first like to thank the Bair Ranch Foundation for their support for this project. To Dr. Carl Yeoman, to you I owe everything. Thank you for all your continuous encouragement, for believing in me even as an undergrad, and for offering me the opportunity to grow and excel in my career. Dr. Joanna-Lynn Borgogna, you saw my potential before I ever recognized it myself, and for that I will be forever grateful. You have become such an incredible friends and mentor that I will always look up to. I can only hope to one day be as kind and supportive to students as you have been towards me. To Dr. Craig Carr, thank you for being such an awesome Co-PI and for continuing to encourage me even when times were a little rocky. Dr. Lance McNew, thank you for allowing me to join in on your ecology research and for helping me navigate my future transition into wildlife ecology at University of Florida. Eri, my fellow lab mate and best friend; we've been through the entire graduate student experience together and I will forever cherish our lunch dates to escape lab and class work just to enjoy each other's company. Finally, to all my friends and family for their love and support when I needed it most, thank you!

TABLE OF CONTENTS

TABLE OF CONTENTS - CONTINUED

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

ABSTRACT

Tall larkspur (*Delphinium* spp.) in the western United States present a serious toxicity danger to rangeland cattle. Consumption of Methyllycaconitine (MLA), the toxic alkaloid in larkspur plants, can cause annual losses of 5–15% of range cattle in grazing pastures with sufficient larkspur. With the wide distribution and abundance of larkspur, wild ruminants in Montana likely encounter tall larkspur while foraging; however, no evidence suggests they are negatively affected by MLA's toxic effects. Therefore, we evaluated: i) whether alkaloids in Delphinium spp., and MLA specifically degraded within ruminal specimens collected from Montana's wild ruminant species over 48 h using *in vitro* incubations; ii) whether observed degradative activities were abiotic, or mediated by either the fungal or non-fungal (mostly bacterial) residents of the ruminal microbiota in wild ruminant specimens; and iii) if representative microbial isolates individually possessed the ability to degrade MLA within *in vitro* incubations. Rumen samples were collected from wild ruminant species during the 2019 and 2020 hunting seasons using legal methods by volunteer hunters. In all assays, total alkaloid was measured spectrophotometrically, and MLA by High-Performance Liquid Chromatography Mass Spectrometry (HPLC) from initial and final incubations. Our results demonstrated that, with the exception of white-tailed deer, all wild ruminant species exhibited variable degradative abilities in both total alkaloid ($P< 0.001$) and MLA ($P< 0.001$) assays and that such degradation was predominantly mediated by ruminal fungi. Additionally, screening of 15 fungal isolates, representing 10 known genera and 2 isolates of unknown taxonomic identity each obtained from herbivorous hosts, determined all were capable of degrading MLA to some extent. Fungal isolates obtained from wild ruminants exhibited greater degradative activity, with *Aestipasuomyces* R5 isolated from wild sheep degrading 71% of MLA ($P<0.001$). Overall, our results indicate that degradation of both total alkaloid and MLA-specifically occurs within the gastrointestinal tract of Montana's wild ruminants and that it is largely influenced by fungal activity. Additionally, fungal strains isolated from wild ruminants are capable of degrading MLA and have the potential to be further used as a direct fed microbial to rangeland cattle as an optimal way to mitigate larkspur toxicosis.

CHAPTER ONE

INTRODUCTION

In 2002, in the western United States alone, an estimated $500 million in annual losses was reportedly due to poisoning plants (Holchek, 2002). In western North America, larkspur (*Delphinium spp.*) is widely distributed and poses a serious toxicity and mortality danger for range cattle (Welch et al. 2015). Larkspur poisoning in the western U.S. causes more deaths to cattle than any other plant, disease, or predator (Aldous 1917, Nielsen et al. 1987). Annual herd mortality due to larkspur toxicity can be as high as 10%, and result in millions of dollars in economic losses (Pfister et al. 1996).

The most common tall larkspur species in Montana is *Delphinium occidentale*, which contains various norditerpenoid alkaloids including the highly toxic Methyllycaconitine alkaloid (MLA). MLA alkaloids are responsible for the toxic effects on livestock poisoned by larkspur (Panter et al. 2002, Pfister et al. 1996). Upon ingestion, MLA causes neuromuscular paralysis by blocking neuromuscular junctions in skeletal muscle and the brain (Aiyar et al. 1979, Dobelis et al. 1999). Neuromuscular junction inhibition by MLA occurs via direct competitive antagonism of the nicotinic acetylcholine receptors, affecting both the sympathetic and parasympathetic systems (Aiyar et al. 1979, Dobelis et al. 1999).

The effects of tall larkspur are of great concern for western livestock producers due to costs associated with cattle losses and mitigation. Management options for livestock producers to mitigate larkspur toxicosis in their cattle include restricting cattle from grazing in areas with abundant larkspur growth, using herbicidal control, or grazing sheep in ranges before cattle due to sheep's increased resistance to toxicity (Welch et al. 2015).

Much previous research has demonstrated MLA to be stable and undegraded in the rumen of domestic cattle (Majak, 1993; Knight, 1997). However, similar research has not been performed on other ruminant species. While it is unknown whether wild ruminants feed on larkspur plants specifically, we may assume these animals are grazing on or near larkspur plants while foraging due to the wide distribution and abundance of the plants. However, unlike cattle, these species may not be as negatively affected by the toxic alkaloid; suggesting they may be less sensitive to MLA. Therefore, we hypothesized that wild ruminants must possess a metabolism that is capable of degrading MLA. Furthermore, because many gastrointestinal tract microorganisms are capable of detoxifying toxic components in the diet, we additionally hypothesized that a microbial metabolism may be responsible for the degradation of MLA.

The aims of this thesis were to determine whether degradation of alkaloid extracted from *Delphinium* spp. may occur within the rumen of wild ruminants, to investigate the total microbial influence on alkaloid degradation, and whether individual microbial isolates possess the ability to degrade toxic alkaloids in hopes of further exploitation and use as a direct fed microbial to cattle as means to protect against larkspur toxicosis. In **Chapter 2** we review larkspur toxicosis in cattle and the potential of microbially mediated alkaloid degradation in wild ruminants, focusing on alkaloids and their toxic effects, the role of rumen microorganisms in xenobiotic metabolism, larkspur toxicity in ruminants, and current management recommendations for mitigating larkspur toxicosis in cattle. In **Chapter 3**, we cultured and assayed ruminal foregut samples from Montana's wild ruminant populations to determine the percent degradation of toxic alkaloids present in tall larkspur. Similarly, in **Chapter 4,** we aimed to refine our understanding of the role of microbes in degradation of the toxic alkaloids in tall larkspur plants by performing assays to determine if total

alkaloid- and MLA-degradation results from bacterial, fungal, or from abiotic factors found in the rumen contents of wild ruminant species from Montana. Finally, in **Chapter 5**, we discuss our collective observations and their implications for range cattle management.

References Cited

Aiyar, V.N., M.H. Benn, T. Hanna, J. Jacyno, S.H. Roth, and J.L. Wilkens. 1979. The principal toxin of Delphinium brownie Rybd., and its mode of action. Experientia, 35, 1367.

Aldous, A.E., 1917. Eradicating tall larkspur on cattle ranges in national forests. USDA Farmers Bull. 826, Washington, DC.

Dobelis, P., J.E. Madl, J.A. Pfister, G.D. Manners, J.P. Walrond. 1999. Effects of *Delphinium* alkaloids on neuromuscular transmission. J. Pharmacol. Exp. Ther. 291, 538-546.

Holechek, J.L. 2002. Do most livestock losses to poisonous plants result from "poor" range management? J. Range Management 55:270-276.

Knight, A. P., and J.A. Pfister, J. A. 1997. Larkspur poisoning in livestock: Myths and misconceptions. Society for Range Management, 19(3), 10-13.

Majak, W. 1993. Alkaloid levels in a species of low larkspur and their stability in rumen fluid. Society for Range Management, 46(2), 100-103.

Nielsen, D.B., and M.H. Ralphs. 1987. Larkspur economic considerations. In: James, L.F., Ralphs, M.H., Nielsen, D.B. (Eds.), Ecology and Economic Considerations of Poisonous Plants. Westview Press, Boulder, *CO*, pp. 119–129.

Panter, K. E., G.D. Manners, B.L. Stegelmeier, S. Lee, D.R. Gardner, M.H. Ralphs, J.A. Pfister, and L.F. James. 2002. Larkspur poisoning: toxicology and alkaloid structure-activity relationships. Biochemical systematics and ecology, 30(2), 113-128.

Pfister, J. A., G.D. Manners, D.R. Gardner, K.W. Price, and M.H. Ralphs. 1996. Influence of alkaloid concentration on acceptability of tall larkspur (delphinium spp.) to cattle and sheep. J. Chemical Ecology, *22*(6), 1147-1168.

Welch K.D., D.R. Gardner, D. Cook, and J.A. Pfister. 2015. The effect of administering multiple doses of tall larkspur (*Delphinium barbeyi*) to cattle. J. Animal Science, 93(8), 4181-4188.

CHAPTER TWO

LITERATURE REVIEW

Alkaloids and Their Effects on Large Ruminants

Alkaloids and Their Role in Plant Defense

Alkaloids are cyclic organic compounds containing nitrogen in a negative oxidation state (Benn, 1983). Alkaloids are not widely distributed among living organisms but are the most common nitrogen-containing secondary metabolites of plants. (Benn, 1983). Plant-derived alkaloids are involved in various aspects of plant metabolism, catabolism, and physiology (Matsuura and Fett-Neto, 2015). However, the ability of plant-derived alkaloids to act as a defense against herbivory is of interest in research related to toxicosis in large ruminants.

Defensive strategies to prevent herbivory require plants to render themselves inedible by producing secondary compounds that affect its taste or smell, or the growth, development, and survival of the host. Alkaloids are an efficient means of defense due to their potent toxic capabilities. Alkaloids are derived from amino acids that are required for more important processes such as protein synthesis. Because of biosynthetic competition, production of alkaloids is often limited. To offset their low quantity, alkaloids are typically highly potent and may illicit damaging effects when only small amounts of plant tissue are ingested (Palo and Robbins, 1991). Toxicity from alkaloids can have a variety of effects on herbivores, including inducing liver and brain damage, teratogenicity, hormonal and reproductive interference, or fatality (Palo and Robbins, 1991).

Among plant alkaloids, the diterpenoids are widely distributed in the two plant genera *Aconitum* and *Delphinium* from the *Ranunculaceae* family. Diterpenoid alkaloids and are understood to be a naturally occurring nitrogenous base with a nitrogen-functionalized skeleton that is formed from a C_{20}-terpenoid precursor (Benn, 1983). Nitrogen-containing metabolites are relatively restricted in plants as secondary compounds and are only found in approximately 20% of angiosperm species (Palo and Robbins, 1991).

Alkaloid Poisoning in Livestock and Wild Ruminants

Plants containing toxic secondary compounds often have detrimental consequences on livestock, causing substantial losses in many instances. Anagyrine, a quinolizidine alkaloid found in the *Lupinus* species, are teratogenic in cattle and are thought to inhibit fetal movement and lead to crooked calf syndrome (Green et al. 2019; Panter et al., 1999). In large doses piperidine alkaloids, also found in certain *Lupinus* species, can be acutely toxic to livestock, and at lower doses may produce fetal deformities (Green et al., 2019; Panter et al., 1990). In western North America, larkspur species containing toxic norditerpenoid alkaloids are responsible for more cattle losses than any other poisonous plant by means of respiratory paralysis (James et al., 1992; Ralphs et al., 1988).

While available information on plant poisoning in wild ruminants is sparse, it is important to understand that toxicity in free-range herbivores can and does occur. In one instance, essential oils that contain terpene compounds were found to be highly unpalatable and disrupt digestion in deer (Fowler, 1983). In addition to cattle, certain wildlife species have also been poisoned eating *Lupinus* species containing certain quinolizidine and piperidine alkaloids (Panter et al., 1990).

Animal Species Variation in Response to Secondary Plant Compounds

Selective pressures for acquiring the ability to manage secondary plant compounds are closely related to the consumption of such chemicals. Therefore, it is reasonable to assume the feeding niches of various herbivorous species may be a determinant used to cope with toxic plant consumption. All ruminant species may be grouped into four feeding niche types, including grazers (25%), browsers, including generalist-, and specialist-browsers (40%), and mixed feeders (35%) each varying in the quantitative gradient of potential secondary plant compound exposure (Shipley, 2001). Grazers generally consume plants containing low levels of secondary plant compounds like grasses and some forbs (Palo and Robbins, 1991). Thus grazers (e.g. cows) and some grass-preferring intermediate mixed feeders (i.e. domestic sheep) likely encounter very small amounts of toxic secondary plant compounds during foraging. Browsers, who generally prefer forbs and shrubs/trees in their diet (e.g., deer, moose), are more likely to consume higher levels of secondary plant compounds (Palo and Robbins, 1991).

Evolutionary and behavioral adaptions to plant toxins in wild ruminant species that have been exposed to specific plants for many generations may also play a role in their ability to avoid toxicosis (Palo and Robbins, 1991). Because domestic animals are generally confined to fenced rangelands, there has been no opportunity for them to adapt to certain plants. Native wild ruminants are known to possess the capability to safely consume large quantities of poisonous plants native to their habitat; however, poisonings are still possible (Fowler, 1983). Additionally, many wild ruminant species have fastidious grazing habits where they nibble small quantities from multiple plants. This behavioral adaptation enables these species to graze a larger variety of vegetation and minimize the likelihood of consuming enough toxins to constitute a lethal dose (Cheeke, 1998).

Wild ruminants also tend to have large and unrestricted home ranges. By covering more area, these animals can consume a more diverse diet and avoid being confined to feeding on a single toxin-containing plant based on lack of other feed, as sometimes observed with confined domestic ruminants (Cheeke, 1998). Additionally, exposure to toxic plants requires species specific habitat requirements to overlap with the habitat of the toxic plants, both in time and space. Nevertheless, there is little knowledge about whether wild ruminants have evolved specific metabolic defenses against poisonous plants, and it is generally thought their grazing and behavioral adaptions are probably the major limiting factors to poisoning.

Consequences of Plant Poisoning for Livestock Production

The poisoning of livestock by the consumption of plants is a principal cause of direct loss to the livestock industry including livestock death, reduced performance, and reproductive loss. In 2002, in the western US alone, an estimated $500 million in annual losses were reportedly due to poisonous plants (Holechek, 2002). Indirect losses may include costs incurred by livestock operations to prevent direct and/or economic losses due to livestock poisoning by plants. Risk management losses may include fencing to prevent grazing of a certain area, supplemental feeding, altered grazing programs which may inadvertently increase costs or grazing inefficiency, medical costs for incidence of poisoning, and potential forage loss due to the inability to harvest at an adequate time or intensity (James et al., 1992).

Larkspur Toxicity in Ruminants

Larkspur Description and Palatability

In the foothill and mountain rangelands of western North America, larkspur poses a serious toxicity danger for livestock. The larkspur plant is responsible for more cattle deaths in the western U.S. than any other plant, disease, or predator (Aldous 1917, Nielsen et al. 1987). On ranges with abundant grazing of larkspur, the toxic effects can cause a significant amount of cattle loss, ranging greatly from 2%–15% (Pfister et al. 1996).

Larkspur species are divided into three groups based upon height: low, plains, and tall larkspurs. *Delphinium occidentale*, the tall larkspur species most common in Montana, is typically found in high elevation (2,360–2,575 m), snow-covered sites of varied plant communities (Welch et al., 2015). Tall larkspurs are long-lived perennial plants that grow in early summer (Pfister et al., 2014). Flowering stalks grow to 18–24 inches in height and form clusters of buds on each stalk. After bud clusters are fully formed, elongation of the flowering stems begins; this is considered the early flowering stage. Larkspurs reach their maximum height of 36 to 72 inches in height after approximated 50 days (Pfister et al., 2014). Flowering begins when the plant has reached 80% of their maximum height, usually beginning mid-to late July. Later in the season (August-September), seed pods will form from mature flowers, dry, shatter, and disperse seeds. Tall larkspurs may be identified by their distinct spur behind a blue flower that is approximately ¾ inches long (Pfister et al., 2014). These plants have multiple large hollow stems, with wide-bladed leaves growing from slender stalks. The leaves of are easily identifiable as they usually consist of 3 to 7 deeply toothed lobes in a palm shape. Mature plants may reach up to 36 to 72 inches in height (Pfister et al., 2014).

Larkspur plants contain multiple norditerpenoid alkaloids that can be categorized into two predominant types: the highly toxic N-(methylsuccinyl) anthranoyllycoctonine (MSAL)-type and the non-MSAL-type. The alkaloid thought to be most responsible for toxic effects on livestock is methyllycaconitine (MLA; Panter et al., 2002; Pfister et al., 1996). The concentration of MLA varies significantly based on growth stage of larkspur, but may also differ based on species, site, and year of growth (Cook et al., 2011). Generally, alkaloid toxicity is highest during the immature stages of budding and stem elongation (Pfister et al., 1999). Alkaloid concentrations begin to decrease as the plant matures into the flower stage. By the time larkspur flowers transform into pods, alkaloid concentration is minimal (Pfister et al., 1999).

A negative correlation exists between alkaloid concentration and palatability for cattle with palatability decreasing as alkaloid concentrations increase (Pfister et al., 1988). A toxic window has been proposed based on these observations. The most critical period when many animals are poisoned is the 4–5-week period from bud elongation to the formation of mature pods (Pfister et al., 1988).

Methyllycaconitine Alkaloid Structure and Metabolism

Methyllycaconitine is a norditerpenoid alkaloid comprised of a tertiary amine, two tertiary alcohols, four methyl ether groups, and an ester based on anthranilic acid and methylsuccinic acid (Panter et al., 2002). In herbivores, MLA is rapidly absorbed from the digestive tract; however, the rate at which it is absorbed and how it is metabolized and excreted from the animal is poorly understood. Structural degradation of the MLA molecule to mitigate toxic effects is thought to rely on the hydrolysis of the C-18 ester group (Benn and Jaycno, 1983). The resulting amino-alcohol,

lycoctonine, has been found in many instances to be significantly less toxic than its precursor (Benn and Jaycno, 1983; Panter et al. 2002). MLA is believed to remain undegraded in both domestic cattle and sheep, suggesting these species do not possess the metabolic ability to degrade and digest the methyllycaconitine alkaloid (Welch, 2006).

Larkspur Toxicity in Cattle

When the clinical signs of larkspur poisoning were first described, the results of toxicity were thought to be due to neuromuscular blockade (Dozortseva-Kubanova., 1959; Aiyar et al., 1979). It is now known that the methyllycaconitine alkaloid causes neuromuscular paralysis by blocking post-synaptic neuromuscular junctions (Benn and Jacyno, 1983). Such inhibition by MLA occurs via direct competitive antagonism of the neurotransmitter acetylcholine specifically acting at the α1nicontic sites, effecting both the sympathetic and parasympathetic systems (Aiyar et al., 1979; Dobelis et al., 1999). The risk of subclinical toxicosis for cattle is considered mild to moderate after ingesting 8-11 mg of toxic alkaloids per kilogram body weight (Pfister et al., 1996) with only 2 mg / Kg being the LD_{50} for MLA.

The result of toxicity in cattle is nerve and muscular paralysis (Aiyar et al., 1979); clinical signs include muscular weakness and trembling, straddled stance, respiratory difficulty, exercise intolerance, periodic collapse, and ultimately death from respiratory failure or regurgitation aspiration (Panter et al., 2002; Olsen et al., 1978). Signs of poisoning begin with uneasiness, and apparent stiffness. The animal collapses suddenly, usually due to buckled forelimbs. After a period of rest, the animal may be able to stand but obvious signs of weakness and vomiting can persist. If vomiting, death will often occur as a result from aspiration and asphyxiation. Effects of larkspur

poisoning from MLA inhibition of nAChRs are exacerbated by exertion; avoidance of additional stressors or disturbances is advised (Panter et al., 2002).

Responses to plants containing norditerpene alkaloids, such as larkspur, can vary in cattle depending on biological factors such as breed, sex, and age (Green et al. 2014, 2019). Such factors can affect the arrangement of the alkaloids in the bodies of cattle, causing varying reactions to the toxicity of larkspur alkaloids. Therefore, it is important to consider these factors in livestock as there is potential to influence the molecular arrangement of veterinary drugs. Research suggests that there is a significant difference between cattle breed responses to larkspur consumption (Green et al., 2014). When orally dosed with a MSAL-type alkaloid, a 2.3-fold difference in exercise tolerance was observed between Angus and Hereford breeds and an even greater difference between Herefords and the two dairy breeds, Holstein and Jersey, which appeared to be much more resistant to poisoning (Green et al., 2014). However, all breeds of cattle tested had individuals that were either resistant to larkspur poisoning, or nearly resistant. These differences can likely be attributed to an individual animal's genetic background that may influence resistance to larkspur poisoning (Heaton et al., 2001).

There is also evidence that suggests age can play an important role in cattle responses to larkspur. Grazing studies conducted in pastures containing larkspur suggest that young heifers are likely to consume more larkspur than mature cows and often suffer from more severe clinical signs of poisoning (Pfister and Gardner, 1999; Pfister et al, 2011). Further laboratory studies have been conducted to confirm that younger cows are more susceptible to larkspur poisoning (Green et al., 2018). Ten Angus steers were fed an oral dose of MSAL-type alkaloids as yearlings and again at two years. Concentrations of two larkspur alkaloids, deltaline and methyllycaconitine, were then

measured from serum samples taken at each time (Green et al., 2018). Serum concentrations of both alkaloids were significantly less ($P<0.05$) in the samples taken when the steers were two years old compared to the samples taken as yearlings. These results suggest that as an Angus steer ages, some physiological changes occur such that the toxicokinetics of the alkaloids are altered (Green et al., 2018).

Sex may also affect cattle susceptibility to larkspur poisoning. Recent research conducted with three groups of Angus cattle: bulls, steers, and heifers demonstrated significant sex-dependent differences in larkspur resistance (Green et al., 2019). The cattle were dosed with MSAL-type alkaloids and serum samples obtained to measure the concentration of alkaloids present. Additionally, cattle were observed for obvious evidence of poisoning. Angus heifers were found to suffer from extensive clinical signs of poisoning including exercise intolerance compared to both steers and bulls. Angus heifers were associated with a 3.3-fold increased relative risk of a zero-walk time, suggesting a much greater risk of larkspur poisoning. However, when comparing serum alkaloid concentration of the three groups, heifers and bulls had higher serum methyllycaconitine concentrations than did steers but heifers and bulls were not significantly different (Green et al., 2019). While toxicokinetic differences between males and females have been well documented and are thought to be due to sex-dependent release of growth hormones by the pituitary gland (Holloway et al., 2006), these results emphasize the importance of sex related issues regarding grazing cattle on rangelands with abundant larkspur.

Susceptibility to larkspur toxicoses has been demonstrated to vary among domesticated ruminants. When dosed with *Delphinium* spp. plant material containing sublethal amounts of alkaloids, domesticated goats and sheep demonstrated minimal to no clinicals signs of poisoning

(Welch et al., 2016). Comparisons of serum MLA concentrations made between goats, sheep, and cattle showed that alkaloid concentration was significantly higher in cattle than in both goat and sheep at 96 hours after dosing (Welch et al., 2006). Due to their ability to degrade MLA at a higher rate than cattle, both domestic goats and sheep are believed to possess an increased resistance to poisoning by larkspur. Similar research has not yet been performed to assess degradation of toxic alkaloids in other ruminant species.

Current Management Consideration

Current management recommendations for mitigating larkspur toxicosis in cattle are based on potential concentrations of total alkaloids and the growth stage of the plants. On ranges with abundant larkspur, increased management in terms of cost and labor are required for producers. Palatability for larkspur is generally low before the plant begins to produce elongated stalks (Pfister, 1988). Producers may take advantage of this low-risk period by grazing before larkspur begin to flower. Risk of toxicosis is highest during the flowering stage to when pods begin to shatter; at this time producers should consider removing cattle from infested ranges. After pods have shattered, cattle may be returned to the pastures for late season grazing (Pfister, 1988).

Controlling larkspur by herbicide application is reasonable if ranges are infested with dense patches. When applied during the immature growth stages (vegetative, bud, and flowering), Picloram (0.9 Kg / acre) is effective at killing larkspur while maintaining grass cover (Pfister et al., 2014). Herbicides Escort (0.02 Kg / acre) and Cimmaron X-tra (0.02 Kg / acre) are other alternative herbicides shown to also eliminate larkspur (Pfister et al., 2014). Lastly, livestock producers may consider grazing sheep on larkspur infested fields before cattle. Both domestic goats and sheep are believed to possess an increased resistance to poisoning by larkspur (Welch et

al., 2006). Normally, sheep do not eat larkspur during the early growing season (Pfister et al., 2014). However, the presence of sheep grazing within dense larkspur patches decreased cattle consumption by up to 50% (Pfister et al., 2014). By trailing or bedding sheep through larkspur leads to more broken stems due to trampling. Broken stems dry out more quickly than unbroken stems and lessens the potential for consumption.

A variety of treatment options are available for animals poisoned by larkspur. Cattle that have been recumbent for at least 30 minutes may be treated neostigmine injected intramuscularly (0.04 mg / Kg body weight, IM). (Pfister et al., 2014). Neostigmine works by inhibiting the hydrolysis of acetylcholine and supports cholinergic action by facilitating impulse transmissions across neuromuscular junctions (NCBI). Cattle who are recumbent for less than twenty minutes will likely survive and should be left alone if they are resting on their sternum, head up, and there is no onset of bloating (Pfister et al., 2014). Bloating occurs quickly after larkspur poisoning because the belching mechanism in cattle becomes paralyzed (Pfister et al., 2014). Dosing with anti-bloating medication may be beneficial. Effects of larkspur poisoning from MLA inhibition of nAChRs are exacerbated by exertion; avoidance of additional stressors or disturbances is advised (Panter et al., 2002). It is most important to not stress the animal, as stressed animals often do not recover.

Rumen Microbial Role in Xenobiotic Metabolism

The rumen microbial community (microbiota) comprises approximately 10^{10} - 10^{11} bacteria/mL, 10^{4} -10^{6} protozoa/mL, and 10^{3} - 10^{7} fungi/mL as well as 10^{7}-10^{9} viruses that have all been shown to play important roles in the nutrition and health of animals (Chaucheyras-Durand et al., 2014). The gastrointestinal tract (GIT) microbiota of ruminants synergistically aids in the

digestion of fibrous feed, converting otherwise indigestible nutrients into energy (i.e. short-chain fatty acids) and nitrogen sources (i.e. microbial protein; Kamra, 2005). Diversity within the rumen is believed to be significantly influenced by diet composition (Chaucheyras-Durand et al., 2014), but also by host genetics (Benson et al., 2010) and other environmental factors (Uyeno et al., 2010).

Within the rumen, anaerobic fungi are among the most efficient fiber degraders, making substantial contributions despite their small percentage of total biomass (Kolattukudy, 1985). Fungi produce both proteins that elicit mechanical forces (e.g. expanism) as well as enzymatic activities (e.g. glycoside hydrolases) that enable them to efficiently penetrate fibrous plant cell walls and break down recalcitrant plant polysaccharides (Kolattukudy, 1985). Their role in plant degradation is considered by some to be more significant than any other microorganisms due to their increased ability to produce enzymes capable of degrading difficult fibers.

The ruminant digestive tract, specifically the rumen, is a potential site for detoxification mediated by microorganisms. Ruminants are generally more resistant to plant toxins than hindgut herbivores because of their ability to inactivate and degrade toxins within the rumen. One example of rumen detoxification is that of mimosine, a toxin found in the *Leucaena leucocephala* plant species. In Australia, leuceana can be toxic to domestic ruminants; however, in places such as Hawaii and Indonesia, Leucaena does not negatively affect wild ruminants due to their possession of mimosine-degrading rumen microorganisms (Hammond, 1995). The bacterium *Synergistes jonesii* was previously isolated from Hawaiian goats found to be resistant to Leucaena toxicosis (Hammond, 1995). *Synergistes jonesii* was found to be capable of degrading mimosine, and when provided to cattle eliminated leucaeuna toxicosis (Hammond, 1995).

In summary, larkspur toxicosis has shown to have detrimental consequences on range livestock, causing substantial losses in most cases. Current management options to mitigate risk of larkspur toxicosis are limited and require either intensive labor, increased expenditures, or loss in grazing potential. MLA toxicity effects are variable among domesticated ruminants; however, no further research has studied MLA toxicity within wild ruminants. Wild ruminants that follow a browser type feeding niche are commonly exposed to toxic secondary plant compounds and likely possess protective mechanisms against toxicity through microbial and fungal degradation. Ruminal inoculation into livestock with rumen content of adapted animals may be considered to establish ruminal microbial populations capable of toxic alkaloid degradation within domesticated ruminants.

References Cited

Aiyar, V.N., M.H. Benn , T. Hanna, J. Jacyno, S.H. Roth, and J.L. Wilkens. 1979. The principal toxin of Delphinium brownie Rybd., and its mode of action. Experientia, 35, 1367.

Aldous, A.E., 1917. Eradicating tall larkspur on cattle ranges in national forests. USDA Farmers Bull. 826, Washington, DC.

Benn, M.H., and J.M. Jacyno. 1983. The toxicology and pharmacology of the diterpenoid alkaloids in Alkaloids: Chemical and Biological Perspectives. Pelletier, S.W., ed Wiley, New York, pp. 153-210.

Benson, A. K., S. A. Kelly, R. Legge, F. Ma, S. J. Low, J. Kim, M. Zhang, P. L. Oh, D. Nehrenberg, K. Hua, S. D. Kachman, E. N. Moriyama, J. Walter, D. A. Peterson, and D. Pomp. 2010. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc. Natl. Acad. Sci. USA 107:18933–18938. http://dx.doi.org/10.1073/pnas.1007028107

Chaucheryras-Durand, F., and F. Ossa. 2014. Review: The rumen microbiome: Composition, abundance, diversity, and new investigative tools. The Professional Animal Scientist. 30:1-12.

Cheeke, P.R. 1998. Natural Toxicants in feeds, forages, and poisonous plants. Interstate Publication Inc., Danville, IL.

Cook, D., B.T. Green, K.D. Welch, D.R. Gardner, J.A. Pfister, and K.E. Panter. Comparison of the toxic effects of two duncecap larkspur (*Delphinium occidentale*) chemotypes in mice and cattle. Am. J. Veterinary Research. 72(5):706-714.

Dobelis, P., J.E. Madl, J.A. Pfister, G.D. Manners, and J.P. Walrond. 1999. Effects of Delphinium Alkaloids on Neuromuscular Transmission. The journal of Pharmacology and Experimental Therapeutics. 291(2):538-546.

Dozortseva-Kubanova, P.M. Farmakol. Toksikol. 1959. *22:* 30.

Fowler, M.E. 1983. Plant Poisoning in Free-living Wild Animals: a Review. J. Wildlife Diseases. 19(1): 34-43.

Green, B.T., K.D. Welch, J.A. Pfister, C.G. Chitko-McKown, D.R. Gardner, and K.E. Panter. 2014. Mitigation of Larkspur Poisoning on Rangelands Through the Selection of Cattle. Rangelands. 36(1): 10-15. doi: 10.2111/RANGELANDS-D-13-00031.1

Green B.T., D.R. Gardner, D. Cook, J.A. Pfister, K.D. Welch, and J.W. Keele. 2018. Age-dependent Intoxication by larkspur (*Delphinium*) In Angus Steers. Toxicon 152: 57-59.

Green, B.T., S.T. Lee, D.R. Gardner, K.D. Welch, and D. Cook. 2019. Bioactive Alkaloids from Plants Poisonous to Livestock in North America. Isr. J. Chem. 59: 351-359.

Hammond, A. C. 1995. Leucaena toxicosis and Its control in ruminants. *J. Anim. Sci.*, *73*: 1487-1492.

Heaton, M.P., C.G., Chitko-McKnown, W.M. Grosse, J.W. Keele, J.E. Keen, and W.W. Laergreid. 2001. Interleukin-8 haplotype structure from nucleotide sequence variation in commercial populations of U.S. beef cattle. Mamm. Genome 12: 219-226.

Hobson, P.N., and C.S. Stewart. 1977. The Rumen Microbial Ecosystem. Chapman & Hall, New York, 719pp.

Holechek, J.L. 2002. Do most livestock losses to poisonous plants result from "poor" range management? J. Range Management 55:270-276.

Holloway, M.G. E.V. Laz, and D.J. Waxman. 2006. Codependence of growth hormone-resposive, sexually dimorphic hepatic gene expression on signal transducer and activator of transcription 5b and hepatic nuclear factor 4alpha. Mol. Endocrinol. 20:647-660

James, L.F., D.B. Nielsen, and K.E. Panter. 1992. Impact of poisonous plants on the livestock industry. J. Range Management. 45:3-8.

Kamra, D.N. 2005. Rumen Microbial Ecosystem. Current Scicnce. 89(1): 124-135.

Knight, A. P., and J.A. Pfister. 1997. Larkspur poisoning in livestock: Myths and misconceptions. *Society for Range Management*, *19*(3): 10-13.

Kolattukudy, P.E. 1985. Enzymatic Penetration of the Plant Cuticle by Fungal Pathogens. Ann. Rev. Phytopatology. 23: 223-250.

Matsuura HN, Rau MR, Fett-Neto AG. 2014.Oxidative stress and production of bioactive monoterpene indole alkaloids: biotechnological implications. Biotechnol Lett. 36:191–200.

Nielsen, D.B., and M.H. Ralphs. 1987. Larkspur economic considerations. In: James, L.F., Ralphs, M.H., Nielsen, D.B. (Eds.), Ecology and Economic Considerations of Poisonous Plants. Westview Press, Boulder, *CO*, pp. 119–129.

Olsen, J.D. 1978. Larkspur Toxicosis: A Review of Current Research. Academic Press, pp. 535-543.

Palo, R.T., and C.T. Robbins. 1991. Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton, FL. Pp. 103-115.

Panter, K.E., T. D. Bunch, R. F. Keeler, D. V. Sisson, and R. J. Callan. 1990. J. Toxicol. Clin. Toxicol, 28: 69–83.

Panter, K.E., L. F. James, and D. R. Gardner. 1999. Lupines, poison-hemlock and Nicotiana spp: toxicity and teratogenicity in livestock J. Nat. Toxins, 8: 117–34.

Panter, K.E., G.D. Manners, B.L. Stegelmeier, S. Lee, D.R. Gardner, M.H. Ralphs, J.A. Pfister, and L.F. James. 2002. Larkspur poisoning: toxicology and alkaloid structure-activity relationships. Biochemical Systematics and Ecology. 30(2):113-128.

Pfister, J.A., G.D. Manners, M.H. Ralphs, Z.X. Hong, and M.A Lane. 1988. Effects of Phenology, Site, and Rumen fill on Tall Larkspur Consumption by Cattle. J. Range Management. 41(6): 509-514.

Pfister, J.A., G.D. Manners, D.R. Gardner, K.W. Price, and M.H. Ralphs. 1996. Influence of alkaloid concentration on acceptability of tall larkspur (*Delphinium* spp.) to cattle and sheep. Journal of Chemical Ecology. 22(6):1147-1148.

Pfister, J.A., D.R. Gardner, K.E. Panter, G.D. Manners, M.H. Ralphs, B.L. Stegelmeier, and T.K. Schoh. 1999. Larkspur (*Delphinium* spp.) Poisoning in Livestock. J. Nat Toxins 8: 81-94.

Pfister, J.A., and D.R. Gardner, Consumption of low Larkspur (Delphinium nuttallianum) by Cattle. J. Natural Toxins. 8(1): 27-34.

Pfister, J.A. D.R. Gardner, and E.A. Burritt. 2014. Grazing Tall Larkspur Ranges: A livestock Producer's Decision-making Handbook. Environmental Science.

Ralphs, M. H., J. A. Pfister, and J. D. Olsen. 1988. Reducing larkspur poisoning in cattle on mountain ranges. Utah Science. 50(2): 109-115. [9095]

Shipley, L.A. 2001. Grazers and Browsers: How Digestive Morphology Affects Diet Selection.

Uyeno, Y., Y. Sekiguchi, K. Tajima, A. Tak-enaka, M. Kurihara, and Y. Kamagata. 2010. An rRNA-based analysis for evaluating the effect of heat stress on the rumen microbial composition of Holstein heifers. Anaerobe 16:27–33. http://dx.doi.org/10.1016/j.anaer obe.2009.04.006.

Vilariño M.P., and D.A Ravetta. 2008. Tolerance to herbivory in lupin genotypes with different alkaloid concentration: interspecific differences between Lupinus albus L. and L. angustifolius L. Environ Exp Bot. 63:130–6.

Welch, K.D., D.R. Gardner, B.T. Green, C.A. Stonecipher, D. Cook, J.A. Pfister. 2006 Comparison of the serum toxicokinetics of larkspur toxins in cattle, sheep, and goats. Toxicon. 119:270-273.

Welch, K.D., B.T. Green, D.R. Gardner, D. Cook, and J.A. Pfister. 2015. The effect of administering multiple doses of tall larkspur (*Delphinium barbeyi*) to cattle. 93:4181-4188.

CHAPTER THREE

DEGRADATION OF TOXIC ALKLAOIDS IN *Delphinium occidentale* SPECIES OCCURS WITHIN THE GASTROINTESTINAL TRACT OF MONTANA'S WILD RUMINANTS

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Savannah G. Grace

Contributions: Implementation of study, first draft.

Co-Author: Joanna-Lynn C. Borgogna

Contributions: assistance with study and revisions.

Co-Author: Craig A. Carr

Contributions: study design and study implementation.

Co-Author: Lance B. McNew

Contributions: assistance with study.

Co-Author: Brian Bothner

Contributions: study design and technical support.

Co-Author: Carl J. Yeoman[1]

Contributions: funding, study design, study implementation, revisions.

Manuscript Information Page

S.G. Grace, J.C. Borgogna, C.A. Carr, L.B. McNew, B. Bothner, C.J. Yeoman
Journal of Animal Science
Status of Manuscript:

__x_ Prepared for submission to a peer-reviewed journal
____ Officially submitted to a peer-review journal
____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

American Society of Animal Science

Degradation of Toxic Alkaloids in *Delphinium occidentale* Species Occurs Within the Gastrointestinal Tract of Montana's Wild Ruminants

Savannah G. Grace[1], Joanna-Lynn C. Borgogna[1], Lance McNew[1], Brian Bothner[3], Craig A. Carr[1], Carl J. Yeoman[1,2*]

[1] Department of Animal & Range Sciences, Montana State University, Bozeman, MT, USA
[2] Department of Microbiology & Immunology, Montana State University, Bozeman, MT, USA
[3] Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT, USA

ABSTRACT: Tall larkspur (*Delphinium* spp.) is a plant that grows abundantly in western North America, where it presents a serious toxicity danger to rangeland cattle. Consumption of the toxic alkaloid, methyllycaconitine (MLA) found in tall larkspur causes an estimated loss of 5–15% of rangeland cattle annually. Due to the wide distribution and abundance of larkspur, wild ruminants in western North America likely encounter tall larkspur frequently while foraging; however, there is no evidence that wild ruminant species are negatively affected by larkspur and MLA. We therefore hypothesized that the gastrointestinal microbiota of wild ruminant species might facilitate protection against MLA toxicity. To test this, 68 ruminal foregut samples were collected from pronghorn antelope, mule and white-tailed deer, elk, moose, bighorn sheep, mountain goat, and bison harvested throughout Montana in 2019 and 2020 by volunteer hunters. Rumen samples were assayed for total alkaloid- and MLA-specific degradation activities over 48 h in *in vitro* incubations. Prior to and following incubations, total alkaloid was extracted and measured spectrophotometrically, and MLA was measured by High-Performance Liquid Chromatography Mass Spectrometry (HPLC-MS). All wild rumen specimens, except those collected from white-tailed deer exhibit significant degradation of total *Delphinium* species alkaloids (P<0.001).

Additionally, all wild ruminant species are capable of significantly degrading the major toxic alkaloid MLA (P= 0.001). Neither total alkaloid or MLA degradation varied in relation to sex, region or elevation of harvest, or month or year of harvest. We conclude that a novel microbial metabolism capable of degrading toxic alkaloids, including MLA is common among most of the wild ruminant species in western North America and propose it may be exploited to protect rangeland cattle.

Key words: larkspur, methyllycaconitine, toxicosis, alkaloids

* Corresponding author's email address: carl.yeoman@montana.edu

INTRODUCTION

Tall larkspur (*Delphinium spp.*) poses a serious toxicity danger for livestock and is responsible for more cattle deaths in the western U.S. than any other plant, disease, or predator (Aldous, 1917; Nielsen et al., 1987). *Delphinium occidentale*, one of the tall larkspur species most widely distributed in western North America, is typically found in high elevation, snow-covered sites of varied plant communities (Welch et al., 2015). These plants contain multiple norditerpenoid alkaloids that can be categorized into two predominant types: the highly toxic N-(methylsuccinyl) anthranoyllycoctonine (MSAL)-type and the non-MSAL-type. Of all the alkaloids, the one that is thought to be most responsible for toxic effects on livestock has been identified as methyllycaconitine (Panter et al., 2002; Pfister et al., 1996). Methyllycaconitine (MLA) causes neuromuscular paralysis by blocking post-synaptic neuromuscular junctions (Benn and Jacyno, 1983). Such inhibition by MLA occurs via direct competitive antagonism of the neurotransmitter acetylcholine specifically acting at the α-1 nicontic sites, affecting both the sympathetic and parasympathetic systems (Aiyar et al., 1979; Dobelis et al., 1999). The risk of subclinical toxicosis for cattle is considered mild to moderate after ingesting 8–11 mg of toxic alkaloids per kilogram (Kg) body weight (Pfister et al., 1996), with just 2 mg / Kg being the median lethal dose for MLA.

Gastrointestinal microbes have been shown to degrade toxic secondary plant compounds responsible for animal mortality in a variety of species (Hammond, 1995; Miller et al., 2014). While MLA remains stable and undegraded in the rumen content of domestic cattle and sheep (Majak, 1993; Knight, 1997), it is unknown whether other ruminants are capable of degrading these toxins (Knight, 1997). Due to the abundant distribution of larkspur, wild ruminants in the

western United States regularly forage on or near the plant (**Supplemental Figure 1);** however, there is no evidence to suggest these species are negatively impacted by the toxic alkaloids present in larkspur, perhaps suggesting they may be less sensitive to MLA. Here we seek to determine whether wild ruminants possess a gut microbial metabolism that is capable of degrading toxic plant alkaloids including MLA.

MATERIALS AND METHODS

Evidence of Larkspur Consumption by Wild Ruminants

During the period of study, we set out game cameras on larkspur-dense areas in an attempt to capture incidental use of larkspur by wild ruminants. On several occasion's animals, including elk, mule deer, and moose were captured grazing on or near larkspur (**Fig. S1**), however the resolution of our photos was insufficient to conclusively state that larkspur was consumed.

Plant Material and Sample Extraction

We collected tall larkspur in the early budding stages during May 2020 near Bozeman, MT, USA (45.883553, -110.8805010, 1942.0 m) and again in August 2020 near Big Sky, MT USA (45.319461, -111.388264, 2524.0 m). Samples were dried at 60°C before being ground to < 2 mm. Extract samples were prepared using methods of decoction; a 1:50 (wt: vol) dilution of dried ground larkspur in distilled water was boiled and concentrated for 12 hours. The presence of total alkaloids and concentration of MLA was confirmed by High-performance liquid chromatography mass spectrometry (HPLC-MS) as described below.

Study Population and Sample Collection

Foregut (rumen) samples were collected from seven different species of wild ruminant species by volunteer hunters using legal methods during the designated Montana hunting season (September–December) in the fall of 2019 and 2020. We provided hunters with verbal and written instructions on how to locate and sample the rumen, including field diagrams. Hunters collected rumen samples from pronghorn antelope (*Antilocapra americana*, n=10), mule deer (*Odocoileus hemionus*, n=10), white-tailed deer (*Odocoileus virginianus*, n=10), elk (*Cervus canadensis*, n=8), moose (*Alces alces*, n=10), bighorn sheep (*Ovis canadensis*, n=10), and mountain goat (*Oreamnos americanus*, n=10) species (**Table 1**). Animals were harvested in each hunting region across the state of Montana (designated by Montana's Fish, Wildlife, and Parks service as regions 1–7) and at varying elevations (**Table 1**). Upon harvest, rumen contents were collected by cutting an incision into the rumen wall and collecting both liquid and solid content in a sterile 50 mL polypropylene conical tube. Hunters were asked to place samples in a freezer (-20 °C) as soon as possible after collection and store samples there until they were able to be returned to the laboratory at Montana State University. We discarded any unfrozen samples upon collection from hunters. The time between initial freezing after harvest and collection by a research team member was a maximum of four months. Samples were transported on dry ice and returned to the lab where they were stored at -20 °C until processing. Elk species was represented by only 8 individual samples due to viability concerns or lack of ruminal fluid during hunter collection.

Alkaloid Degradation Assays

Rumen samples were assayed for total alkaloid- and MLA-degradation activities over 48 h in *in vitro* incubations. We modified methods by Mabjeesh and colleagues (2000 to prepare 75

mL of media per sample assay. Briefly, approximately equal parts liquid and solid rumen content from each specimen were diluted 1:5 (final concentration 20% v:v) with a salt solution refined by Mabjeesh and colleagues (2000). The solution was then inoculated with 2 ng/mL of either total alkaloid from larkspur extract or MLA from pure extract (Tocris Biosciences, Minneapolis, USA), purged with CO_2, and incubated at 39 °C for 48 hours. Prior to (T0) and following (T48) incubations, total alkaloid was extracted and measured spectrophotometrically, while MLA concentrations were measured by High-Performance Liquid Chromatography Mass Spectrometry (HPLC-MS).

Total Alkaloid Extraction Total alkaloid concentrations in the larkspur extract and prior to and following assays were determined using spectrophotometric methods (Sreevidya and Mehrotra, 2003). Briefly, 5 mL of the larkspur extract or assay aliquots were acidified to a pH of 2–2.5 using dilute HCl (50%). 2 mL of a DR solution (1.1% w:v bismuth nitrate and 11% w:v potassium iodide) was added and the new solution was centrifuged at 3000 x g for 10 minutes. To ensure the complete precipitation of alkaloids, an additional 500 µL of DR solution was added before a second centrifugation at 3000 x g was performed for 5 minutes. The centrifugate was decanted and the residue was then treated with 2 mL of 1% disodium sulfide solution and centrifuged again (3000 × g for 10 minutes). A further 500 µL of disodium sulfide solution was added and an additional centrifugation (3000 x g for, 5 minutes) was performed. The final centrifugate was dissolved in 2 mL of concentrated nitric acid and diluted to 10 mL with distilled water. 1 mL was then pipetted out and added to 5mL of 3 % thiourea solution. Absorbance was measured at 435 nm. The amount of alkaloid precipitate was obtained by comparison against a bismuth nitrate standard curve as previously described (Sreevidya & Mehrotra 2003).

Methyllycaconitine (MLA) HPLC-MS Sample Preparation and Analysis Assay aliquots were prepared for HPLC-MS analysis as described by Lee and colleagues (2020). Aliquots were centrifuged at 14800 x g for 10 minutes, the supernatant transferred to a new microcentrifuge tube and centrifuged again (14800 x g for 20 minutes). The supernatant (50 µL) was diluted 50:50 with acetonitrile. An aliquot (100 µL) of the dilute assay sample was transferred to a 300 µL autosampler vial for HPLC-MS.

Methyllycaconitine analysis by HPLC was performed following the protocol outlined by Lee and colleagues (2020) with a few modifications. 5uL samples were injected onto a Acquity UPLC® HSS T3 column (100 x 2.1 mm). Alkaloids were eluted with a gradient flow consisting of 5mM ammonium formate, 0.1% formic acid and acetonitrile at a flow rate of 0.500 mL/min. The mobile phase consisted of 97:3, v:v of 5mM ammonium formate and 0.1% formic acid-acetonitrile for 1 minute followed by a linear gradient to a composition of 50% acetonitrile. After 10 minutes the mobile phase was injected using an Agilent 1290 UHPLC (Thermo Scientific, San Jose, CA, USA) with the column eluent connected to an Agilent 6538 Q-TOF (Thermo Scientific, San Jose, CA, USA). With this method, methyllycaconitine (MLA) eluted at 7.6 minutes Chromatographic peaks were analyzed by generating reconstructed HLPC-QTOF chromatograms with the calculated MH^+ calculated weight for MLA ($MH^+ = 683.3538$) with a mass tolerance of 20 ppm.

Statistical Analysis

Total alkaloid and MLA degradation were assayed for 68 wild rumen samples, consisting of 10 individual rumen samples from pronghorn antelope, mule deer, white-tailed deer, moose bighorn sheep, and mountain goat, and 8 samples from elk. Linear mixed-effects regression was

performed on log-transformed alkaloid concentrations over time with a random intercept for sample ID to account for repeated measurements (**Model 1**). To assess MLA-degradation, we adjusted Model 1 such that the response variable was MLA concentration (**Model 2**). Finally, additional models were fit to include a species-by-time interaction term to evaluate whether the associations of either total alkaloid or MLA alkaloid degradation varied by species (**Model 3 and 4).** For all models, the reference for time was 0. We adjusted the reference for model 3 to allow for comparisons of alkaloid degradation over time by each species.

The association between species, sex, harvest elevation, and year and month of harvest and alkaloid and MLA degradation were independently evaluated using an Extra Sums of Squares F-test where a reduced model (**Model 1**) was compared to a model with the sequentially added variables. The variables sex, harvest elevation, and year and month of harvest did not show to have a significant effect on either MLA or total alkaloid degradation ($P > 0.05$).

All analyses were conducted in R Statistical Software (version 1.4). Linear mixed model analyses were performed using the *lmer* function and *lme4* package in R Statistical Software. P values were approximated using Satterthwaite approximation for degrees of freedom and the *afex* package. Statistical significance was defined as confidence intervals excluding 1 and *P* values <0.05.

RESULTS

Degradation Occurrence in All Samples

Significant alkaloid degradation was measured in assays of both total alkaloid and MLA-specific degradation (**Figure 1**). Total alkaloid assays containing *Delphinium* spp. extract degraded on average 37% among all samples ($P<0.001$, 95% confidence interval (CI): 28–43%) (**Figure**

1a). In assays containing pure Methyllycaconitine, MLA degraded on average 48% among all samples (P<0.001, 95% CI:41–53%) (**Figure 1b**).

Degradation by Species

Assays of all wild ruminant specimens, except white-tailed deer, exhibited significant degradation of total alkaloids from *Delphinium* spp. (**Figure 2**). On average, rumen samples from elk and bighorn sheep assays were capable of degrading 48% of total alkaloids (P<0.001, 95% confidence interval (CI): 25–64%, 95% CI: 28–62%, respectively), while moose, mule deer, pronghorn, and mountain goats degraded 42% (P<0.001, 95% CI: 23-56%), 36% (P<0.001, 95% CI: 13–52%), 35% (P=0.005, 95% CI: 14–51%), and 27% (P=0.04, 95% CI: 3–45%) of total alkaloids over the 48 h incubation, respectively. White-tailed deer samples did not significantly alter the total alkaloid concentration (P=0.147).

All seven of Montana's wild ruminant species were capable of significantly degrading MLA, with the average degradation of each species being greater than 30% (**Figure 3**). Mule deer, elk and mountain goats had the largest MLA degradation capabilities with an average of 59% (P <0.001, 95% CI: 45–69%), 56% (P<0.001, 95% CI: 37–69%), and 54% (P<0.001, 95% CI: 40–65%) degradation, respectively, over 48 h. On average, pronghorn and white-tailed deer samples degraded 50% (P<0.001, 95% CI: 33–62%) and 43% (P<0.001, 95% CI: 24–56%). Moose and bighorn sheep had the lowest average degradation across samples at 37% (P=0.007, 95% CI: 14–54%) and 31% (P=0.02, 95% CI: 8–49%).

DISCUSSION

There are several potential limitations to this study. An initial concern is that of sample collection as Samples were collected in the field by hunters during dressing of their wild game harvest so neither sterility, nor rumen capture can be guaranteed. Further, depending on the size of the animal and distance from harvest to home, some specimens may have not been placed in a freezer for several hours to days. Sample collection from hunters by laboratory team members also was not always immediate and in some cases was a maximum of four months after collection. However, delayed collection did not appear to influence results as there was no significant difference in activity by month of harvest. Additionally, rumen microbes are anaerobic and sensitive to oxygen exposure. During sample collection it was impossible to collect ruminal content in an anaerobic manner, so it was possible that some microorganisms active within the rumen may have lost viability prior to being cultured within the assays.

Given the limitations, our results still indicate a rumen-located, likely microbial metabolism exists in wild ruminants, that can degrade the toxic alkaloids found in *Delphinium occidentale* spp., including the highly toxic MLA. The majority of the wild ruminant samples were shown to significantly degrade both total alkaloids as well as MLA specifically.

As means for protection, various plant species have acquired the ability to synthesize alkaloids to deter and prevent attacks from herbivorous predators. To prevent herbivory, plants produce secondary plant compounds, such as alkaloids, to render themselves inedible by either altering their taste or smell, or by directly affecting the growth, development, and survival of the feeder (Palo and Robbins, 1991). In mammalian herbivores, the selective pressures for acquiring the ability to successfully degrade and digest toxic secondary plant compounds are highly

influenced by exposure through consumption of such chemicals, thus is it reasonable to assume feeding niches may determine an individual's ability to mitigate toxicity. Browsers, such as moose and mule deer, who generally prefer to consume forbs and shrubs/trees, are more likely to consume higher levels of secondary plant compounds than that of a grazer (i.e., cattle) whose diets are generally restricted to grasses and leaf tissues (Palo and Robbins, 1991). Our results demonstrating degradation within wild ruminants are consistent with these indications; from the wild ruminants sampled, most species may be defined as either mixed feeders or general browsers (Palo and Robbins, 1991). Each of these species variably demonstrated the ability to successfully degrade alkaloids found in the *Delphinium* plant species. Although it is unknown whether wild ruminants in western United States consume larkspur specifically during foraging, there is reason to believe that due to their specific feeding niches they are ingesting plants with similar alkaloid constitution and are thus capable of successfully metabolizing such compounds.

Nevertheless, due to their increased exposure to plant secondary compounds, ruminants that follow a browser type feeding niche are thought to possess the capability to adapt protective measures through rumen microorganisms to reduce their susceptibility to toxicosis (Loh et al., 2020). The interest in microbially mediated degradation of toxic plant secondary compounds and the ability to potentially isolate such microbes for use in toxicosis mitigation would be of great benefit to the cattle industry. Current management options for larkspur have proven to be both costly and ineffective (Ralphs et al., 1989). Ranchers are required to either defer grazing of larkspur-infested ranges until after flowering when alkaloid concentrations begin to decline, graze sheep before cattle, or control larkspur plants with herbicides that have shown to potentially increase total alkaloid contents with application (Ralphs. et al., 1989). Because many

gastrointestinal tract microorganisms have adapted to detoxify toxic compounds in the diet, as demonstrated by Hawaiian goats and their ability to degrade a toxic compound in Leucaena (Hammond, 1995; Miller, 2014), it may be possible to isolate specific microbial genera from the rumen of wild ruminants and transfer to range cattle to reduce their susceptibility to larkspur poisoning.

TABLES AND FIGURES

Table 1. Individual characteristics of the individual rumen samples collected by volunteer hunters during the 2019 and 2020 hunting seasons. Descriptive characteristics include species, sex, harvest year, harvest elevation, and hunting district.

Individual Characteristics	
	N = 68
Species	
Bighorn sheep	10 (14.7%)
Elk	8 (11.8%)
Moose	10 (14.7%)
Mountain goat	10 (14.7%)
Mule deer	10 (14.7%)
Pronghorn	10 (14.7%)
White-tailed deer	10 (14.7%)
Sex	
Female	22 (32.3%)
Male	45 (66.2%)
Unknown	1 (1.5%)
Harvest Year	
2019	31 (45.6%)
2020	37 (54.4%)
Harvest Month	
September	5 (7.3%)
October	32 (47.0%)
November	25 (36.7%)
December - February	3 (4.4%)
Unknown	3 (4.4%)
Harvest Elevation	
2000-3000	8 (11.8%)
3001-4000	14 (20.6%)
4001-5000	11 (16.2%)
5001-6000	7 (10.3%)
6001-7000	13 (19.1%)
7001-8000	10 (14.7%)
8001-9500	5 (7.3%)
Hunting District	
100	11 (16.2%)
200	7 (10.3%)
300	24 (35.3%)
400	7 (10.3%)
500	8 (11.8%)
600	2 (2.9%)
700	8 (11.8%)

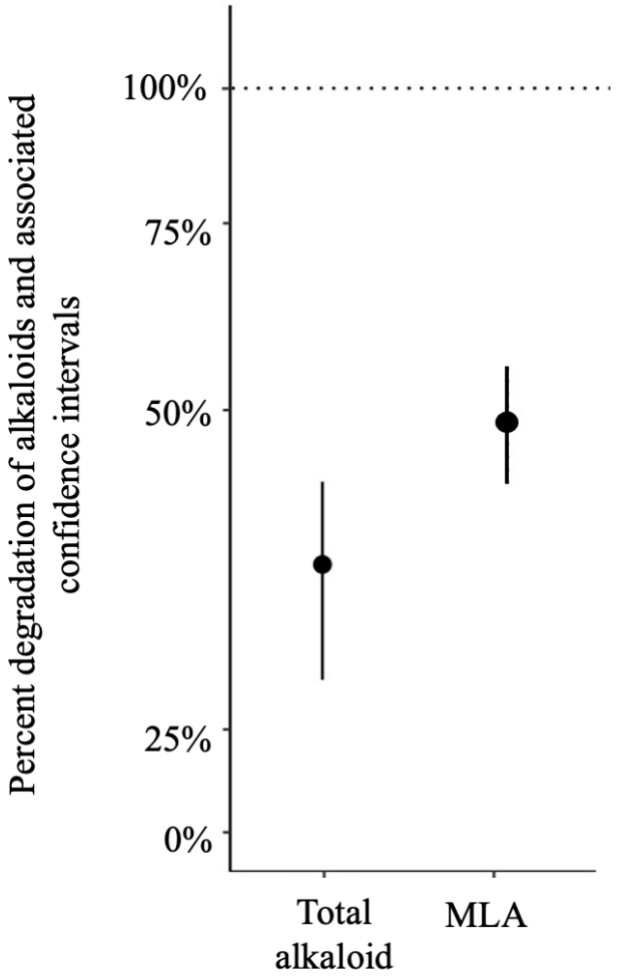


Figure 1. Percent alkaloid degradation across all samples. Total alkaloid represents the percent degradation of total alkaloids from Delphinium spp. extract, while MLA represents the percent degradation of the Methyllycaconitine alkaloid specifically. For each, the dot represents the point estimate comparing total alkaloid concentrations at time 0 and time 48 calculated from exponentiated model coefficients and the associated 95% confidence interval represented by the line.

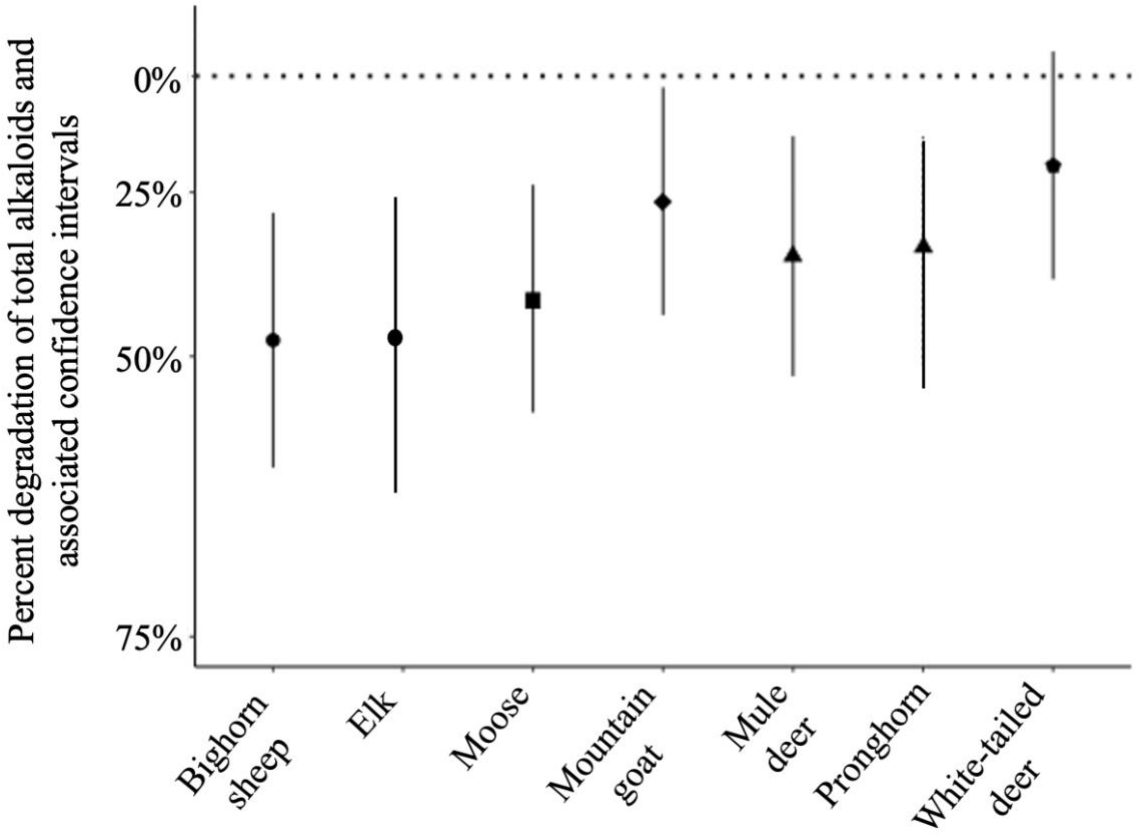


Figure 2. Species specific total alkaloid degradation in assays containing larkspur extract. For each species, the shapes represent the point estimate comparing total alkaloid concentrations at time 0 and time 48 across each species calculated from exponentiated model coefficients and the associated 95% confidence interval represented by the line.

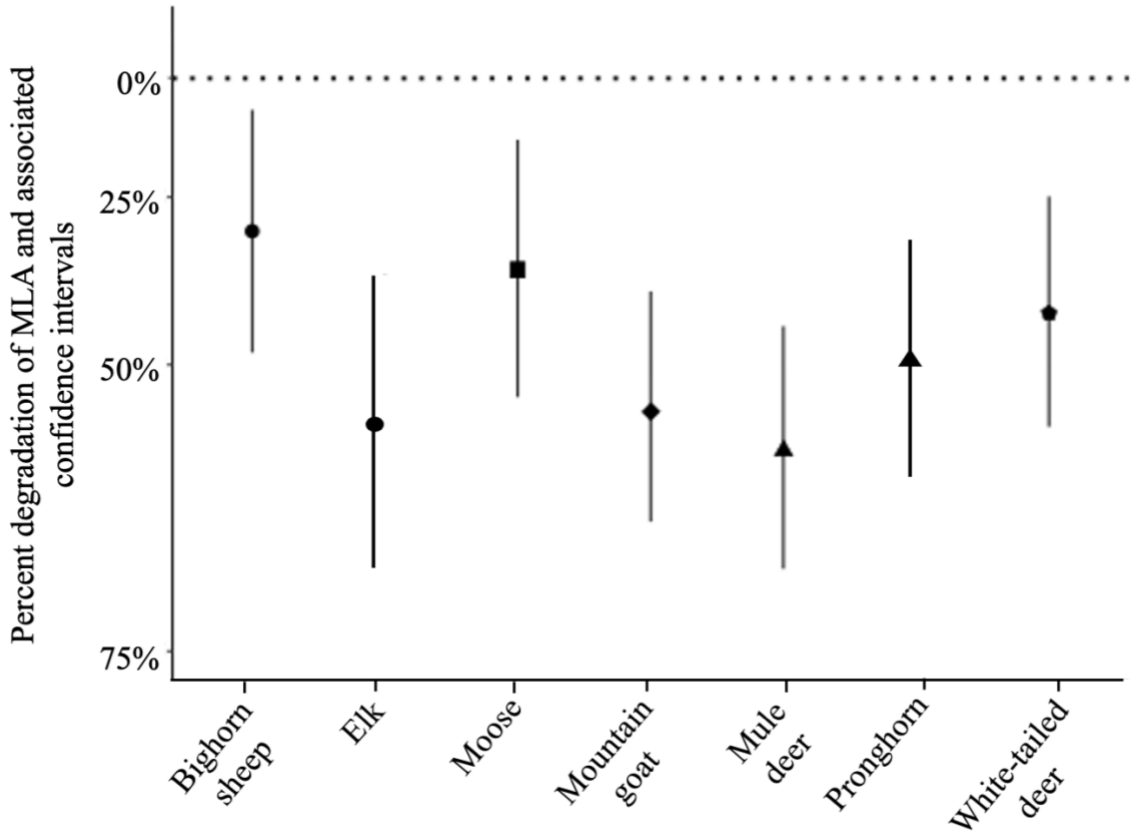


Figure 3. Species specific MLA degradation in assays containing pure methyllycaconitine (MLA). For each species, the shapes represent the point estimates comparing MLA concentrations at time 0 and time 48 across each species calculated from exponentiated model coefficients and the associated 95% confidence interval represented by the line.

SUPPLEMENTAL MATERIAL

Supplementary Figure 1. Photographic evidence of potential larkspur consumption. Photos were captured near Bozeman, MT USA (45.883553, -110.8805010, 1942.0 m) and Big Sky, MT, USA (45.319461, -111.388264, 2524.0 m, USA). Photographs demonstrate evidence of wild ruminant species including moose (top left), mule deer (bottom left), and elk (top and bottom right) grazing on or near tall larkspur.

References Cited

Aiyar, V.N., M.H. Benn , T. Hanna, J. Jacyno, S.H. Roth, and J.L. Wilkens. 1979. The principal toxin of Delphinium brownie Rybd., and its mode of action. Experientia, 35, 1367.

Aldous, A.E., 1917. Eradicating tall larkspur on cattle ranges in national forests. USDA Farmers Bull. 826, Washington, DC.

Aldous, A.E., 1917. Eradicating tall larkspur on cattle ranges in national forests. USDA Farmers Bull. 826, Washington, DC.

Benn, M.H., and J.M. Jacyno. 1983. The toxicology and pharmacology of the diterpenoid alkaloids in Alkaloids: Chemical and Biological Perspectives. Pelletier, S.W., ed Wiley, New York, pp. 153-210.

Dobelis, P., J.E. Madl, J.A. Pfister, G.D. Manners, and J.P. Walrond. 1999. Effects of Delphinium Alkaloids on Neuromuscular Transmission. The journal of Pharmacology and Experimental Therapeutics. 291(2):538-546.

Hammond, A. C. 1995. Leucaena toxicosis and Its control in ruminants. J. Anim. Sci., *73*: 1487-1492.

Hobson, P.N., and C.S. Stewart. 1977. The Rumen Microbial Ecosystem. Chapman & Hall, New York, 719pp.

Knight, A. P., and J.A. Pfister. 1997. Larkspur poisoning in livestock: Myths and misconceptions. *Society for Range Management*, *19*(3): 10-13.

Lee, S.T., K.D. Welch, C.A. Stonecipher, D. Cook, D.R. Gardner, and J.A. Pfister. 2020. Analysis of rumen content and ocular fluid for toxic alkaloids from goats and cows dosed larkspur (*Delphinium barbeyi*), lupine (*Lupinus leucophyllus*), and death camas (*Zigadenus paniculatus*). Toxicon. 176:21-29.

Loh, Z.H. D. Ouwerkerk, A.V. Klieve, N.L. Hungerford, and M.T. Fletcher. 2020. Toxin Degradation by Rumen Microorganisms: A Review. Toxins. 12,664; doi:10.3390/toxins12100664

Mabjeesh SJ, Cohen M, Arieli A. 2000. In Vitro Methods for Measuring the Dry Matter Digestibility of Ruminant Feedstuffs: Comparison of Methods and Inoculum Source J. Dairy Sci. 83: 2289 – 2294

McDougall EI. 1948. Studies on ruminant saliva. I. The composition and output of sheep's saliva. Biochem. J. 43: 99 – 109

Majak, W. 1993. Alkaloid levels in a species of low larkspur and their stability in rumen fluid. Society for Range Management. 46(2): 100-103.

McDougall EI. 1948. Studies on ruminant saliva. I. The composition and output of sheep's saliva. Biochem. J. 43: 99 – 109.

Miller, A.W., K.D. Kohl, M.D. Dearing. 2014. The gastrointestinal tract of white-throated woodrat (Neotoma albigula) harbors distinct consortia of oxalate-degrading bacteria. Applied and Environmental Biology. 80(5). https://doi.org/10.1128/AEM.03742-13

Nielsen, D.B., and M.H. Ralphs. 1987. Larkspur economic considerations. In: James, L.F., Ralphs, M.H., Nielsen, D.B. (Eds.), Ecology and Economic Considerations of Poisonous Plants. Westview Press, Boulder, *CO*, pp. 119–129.

Loh, Z.H. D. Ouwerkerk, A.V. Klieve, N.L. Hungerford, and M.T. Fletcher. 2020. Toxin Degradation by Rumen Microorganisms: A Review. Toxins. 12,664; doi:10.3390/toxins12100664

Palo, R.T., and C.T. Robbins. 1991. Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton, FL. Pp. 103-115.

Panter, K.E., G.D. Manners, B.L. Stegelmeier, S. Lee, D.R. Gardner, M.H. Ralphs, J.A. Pfister, and L.F. James. 2002. Larkspur poisoning: toxicology and alkaloid structure-activity relationships. Biochemical Systematics and Ecology. 30(2):113-128.

Pfister, J.A., G.D. Manners, D.R. Gardner, K.W. Price, and M.H. Ralphs. 1996. Influence of alkaloid concentration on acceptability of tall larkspur (*Delphinium* spp.) to cattle and sheep. Journal of Chemical Ecology. 22(6):1147-1148.

Pfister, J.A. D.R. Gardner, and E.A. Burritt. 2014. Grazing Tall Larkspur Ranges: A livestock Producer's Decision-making Handbook. Environmental Science.

Ralphs, M. H., J. A. Pfister, and J. D. Olsen. 1988. Reducing larkspur poisoning in cattle on mountain ranges. Utah Science. 50(2): 109-115.

Sreevidya, N., S. Mehrotra. 2003. Spectrophotometric Method for Estimation of Alkaloids Precipitable with Dragendorff's Reagent in Plant Materials. Journal of AOAC International. 86(6):1124-1127.

Welch, K.D., B.T. Green, D.R. Gardner, D. Cook, and J.A. Pfister. 2015. The effect of administering multiple doses of tall larkspur (*Delphinium barbeyi*) to cattle. 93:4181-4188.

CHAPTER FOUR

DEGRADATION OF THE TOXIC ALKALOID, METHYLLYCACONITINE BY WILD RUMINANT SPECIES IS PREDOMINANTLY MEDIATED BY RUMEN FUNGI

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

Author: Savannah G. Grace

Contributions: Implementation of study, first draft.

Co-Author: Joanna-Lynn C. Borgogna

Contributions: assistance with study and revisions.

Co-Author: Mostafa Elshahed

Contributions: assistance with study.

Co-Author: Lance B. McNew

Contributions: assistance with study.

Co-Author: Brian Bothner

Contributions: study design and technical support.

Co-Author: Craig A. Carr

Contributions: study design and study implementation.

Co-Author: Carl J. Yeoman

Contributions: funding, study design, study implementation, revisions.

Manuscript Information Page

S.G. Grace, J.C. Borgogna, M.S. Elshaded, L.B. McNew, B. Bothner, C.A. Carr, C.J. Yeoman
Journal of Animal Science
Status of Manuscript:

__x_ Prepared for submission to a peer-reviewed journal
____ Officially submitted to a peer-review journal
____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

American Society of Animal Science

Degradation of the Toxic Alkaloid, Methyllycaconitine by Wild Ruminant Species is Predominantly Mediated by Rumen Fungi

Savannah G. Grace[1], Joanna-Lynn C. Borgogna[1], Mostafa Elshahed[2], Lance McNew[1], Brian Bothner[3], Craig A. Carr[1], Carl J. Yeoman[1,4*]

[1] Department of Animal & Range Sciences, Montana State University, Bozeman, MT, USA
[2] Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, OK, USA
[3] Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT, USA
[4] Department of Microbiology & Immunology, Montana State University, Bozeman, MT, USA

ABSTRACT: Wild ruminants that follow a browser type feeding niche are often exposed to toxic secondary plant compound consumption and presumably possess mechanisms through microbial and fungal mediated degradation that protect from plant induced toxicity. Here we aim to evaluate the rumen microbiota's role in degradation of the toxic alkaloids of *Delphinium* spp. among western U.S. wild ruminant species. Rumen samples from seven wild ruminant species were collected by volunteer hunters and assessed for total alkaloid and MLA-specific degradation over 48 h in *in vitro* incubations. Separate incubations were performed to determine the relative influence of gut bacterial, fungal, and abiotic activities. Initial and final total alkaloid concentrations were extracted and measured spectrophotometrically and MLA by High-Performance Liquid Chromatography Mass Spectrometry (HPLC). Anaerobic fungal isolates cultured from various herbivores were assayed in similar *in vitro* incubations and analyzed to evaluate the percent of MLA degradation by each individual fungal isolate. Degradation of total larkspur alkaloids and MLA was predominantly attributable to a fungal-mediated activity (67.7 ±

38% of total alkaloids and 55.2 ± 36% of MLA-specific degradation). While bacteria appeared to contribute to total alkaloid degradation (25.0 ± 33%), no appreciable contribution to MLA-specific degradation was observed for bacteria. Significant MLA-specific degradation was observed in abiotic samples (32.7 ± 30%). Isolated fungal strains degraded MLA by 31% (P<0.001, 95% CI: 22–38%). Six fungal strains representative of various wild ruminants demonstrated significant MLA degradative capabilities. Overall, these data indicate that MLA may be degraded in wild ruminants by rumen fungi, and that these fungi have the potential to be further exploited as a direct fed microbial to range cattle as means for larkspur toxicosis mitigation.

Key words: larkspur, methyllycaconitine, toxicosis, alkaloids, anaerobic fungi

* Corresponding author's email address: carl.yeoman@montana.edu

INTRODUCTION

Larkspur (*Delphinium* spp.) are widely distributed in the western United States, where they pose a serious danger of toxicity and mortality for range cattle (Welch et, al. 2015). On pastures with high abundance of larkspur, annual herd mortality can be as high as 10% (Pfister et al,, 1996). These losses result in economic losses of millions of dollars in animal deaths, treatment costs, and increased management.

Tall larkspur (*D. occidentale*) contains various N-(methylsuccinyl) anthranoyllycoctonine (MSAL)-type norditerpenoid alkaloids, including the highly toxic Methyllycaconitine (MLA) that is believed to be the most toxic alkaloid in larkspur and is largely responsible for the fatal effects experienced by cattle (Panter et al., 2002; Pfister et al., 1996). Methyllycaconitine affects both the sympathetic and parasympathetic nervous systems by blocking post-synaptic neuromuscular junctions and inhibiting the transfer of the neurotransmitter acetylcholine (Benn and Jacyno, 1983; Aiyar et al., 1979; Dobelis et al., 1999). The result of such inhibition is nerve and muscular paralysis, which ultimately leads to a rapid death in cattle from respiratory failure or regurgitation aspiration (Panter et al., 2002; Olsen et al., 1978).

Options to reduce risk of larkspur related losses are limited and include grazing cattle on larkspur invested fields during times of the season where toxicity is low (May – June), applying herbicides, or grazing sheep on infested pastures as they are believed to possess an increased resistance to poisoning by larkspur (Welch et al., 2016). Each approach requires either intensive labor, increased expenditures, or loss in grazing potential. Additionally, in the case of treating poisoned animals there are few medications capable of reversal; however, administration is usually

required within just 30 minutes of the onset of symptoms (Pfister et al., 2014). More effective alternative measures for mitigating larkspur toxicosis are necessary.

Wild ruminants that follow a browser type feeding niche, feeding mostly on non-grasses, are commonly exposed to toxic secondary compounds, including MLA. These species are thought to possess microbial mechanisms in the rumen that protect against the effects of toxic secondary metabolites (Loh et al., 2020). Ruminal inoculation into livestock with rumen content from adapted animals may be considered to establish ruminal populations capable of secondary plant compound degradation. Previous research has demonstrated the ability to isolate ruminal microbiota in wild Hawaiian goats capable of degrading plant toxins and further mitigating toxicosis when supplemented to domestic livestock (Hammond, 1995). Previously, we showed that degradation of total alkaloid and MLA in *Delphinium* spp. occurs at various levels within wild ruminant species harvested in Montana, USA (Chapter 2; Grace et al. *In preparation*). Here we aim to refine our understanding of the rumen microbiota's role in the degradation of toxic alkaloids from larkspur among wild ruminants. We sought to evaluate the contribution to both total alkaloid- and/or MLA-degradation resulting from bacterial, fungal, or from abiotic factors found in the rumen contents of wild ruminant species from Montana. Additionally, individual anaerobic fungal strains isolated from various herbivores were evaluate for their ability to degrade MLA and potential use as direct fed microbials to rangeland cattle in order to increase cattle resistance to larkspur poisoning.

MATERIALS AND METHODS

Plant Material and Sample Extraction

An enrichment of larkspur alkaloids was prepared as previously described (Chapter 2; Grace et al. *In preparation*). Briefly, plant material and sample extraction occurred as follows:

Budding Tall larkspur (*Delphinium occidentale*) was collected May of 2020 near Bozeman, MT, USA (45.883553, -110.8805010, 1942.0 m) and again in August of 2020 in Big Sky, MT USA (45.319461, -111.388264, 2524.0 m). Samples were dried at 60 °C before being ground to < 2 mm. Extract samples were prepared using methods of decoction; a 1:50 (wt: vol) dilution of dried ground larkspur in distilled water was boiled and concentrated for 12 hours. The presence of total alkaloids and concentration of MLA was confirmed by High-performance liquid chromatography mass spectrometry (HPLC-MS) as described below.

Study Population and Sample Collection

The study population and sample collection has also been previously described (Chapter 2; Grace et al. *In preparation*), in brief 68 foregut (rumen) samples collected from pronghorn antelope (*Antilocapra americana*, n=10), mule deer (*Odocoileus hemionus*, n=10), white-tailed deer (*O. virginianus*, n=10), elk (*Cervus canadensis*, n=8), moose (*Alces alces*, n=10), bighorn sheep (*Ovis canadensis*, n=10), and mountain goat (*Oreamnos americanus*, n=10). Samples were collected by volunteer hunters during the designated Montana hunting season (September-December) in the fall of 2019 and 2020. Hunters were provided verbal and written instructions on how to locate and sample the rumen, including field diagrams. Upon harvest, total rumen contents were collected by cutting an incision into the rumen wall and collecting both liquid and solid content in a sterile 50 mL polypropylene conical tube. Hunters were asked to place samples in a freezer (-20 °C) as soon as possible after collection and store samples there until they were able to be returned to the laboratory at Montana State University. All samples were retrieved from hunters by members of the research team and were observed to be frozen at the time of collection or were otherwise discarded. Collection of samples from hunters and return to the lab took place in

December at the end of each hunting season; time between initial freezing after harvest and collection by a research team member was a maximum of four months. Samples were transported on dry ice and returned to the lab where they were stored at -20 °C until processing.

Alkaloid Degradation Assays

Rumen samples were assayed as previously described (Grace et al. *In preparation*) for total alkaloid- and MLA-degradation activities over 48 h in *in vitro* incubations. Briefly, 75 ml of media were prepared using a slightly modified protocol from that described by Mabjeesh and colleagues (2000), where approximately equal parts liquid and solid rumen content from each specimen were diluted 1:5 (final concentration 20% v:v) with a salt solution described by McDougall (1948) . The solution was then inoculated with 2 ng/mL of either total alkaloid from larkspur extract or MLA from pure extract (Tocris Biosciences, City, Country). To assess fungal-specific activity, an antibiotic cocktail (50 µg/mL kanamycin, 50 µg/mL penicillin, 20 µg/mL streptomycin, and 50 µg/mL chloramphenicol) was added to the final assay solution to inhibit growth of bacteria and methanogenic archaea (Calkins et al., 2016). To assess for any abiotic activity, rumen specimens were autoclaved at 121 °C for 30 minutes prior to addition. Assay solutions were then purged with CO_2 and incubated at 39 °C for 48 hours. Prior to (T0) and following (T48) incubations, total alkaloid was extracted and measured spectrophotometrically, while MLA concentrations were measured by High-Performance Liquid Chromatography Mass Spectrometry (HPLC).

Total Alkaloid Extraction Spectrophotometric methods, as described by Sreevidya and Mehrotra (2003), were followed to determine total alkaloid concentrations in the larkspur extract assays. Briefly, 5 mL of the assay aliquots were adjusted to a pH of 2-2.5 using dilute 12 M HCl (50%). 2 mL of DR solution (1.1% w:v bismuth nitrate and 11% w:v potassium iodide) was added

and centrifuged at 3000 x g for 10 minutes. An additional 500 µL of DR solution was added to ensure the complete precipitation of alkaloids and centrifuged again at 3000 x g was performed for 5 minutes. The centrifugate was decanted, the residue treated with 2 mL of 1% disodium sulfide solution and re-centrifuged (3000 x g for 10 minutes). An additional 500 µL of disodium sulfide solution was added and a subsequent centrifugation (3000 x g for, 5 minutes) was performed. The final centrifugate was dissolved in 2 mL of concentrated nitric acid and diluted to 10 mL with distilled water. 1 mL was then pipetted out and added to 5mL of 3 % thiourea solution. Absorbance was measured at 435 nm. The amount of alkaloid precipitate was obtained by comparison against a bismuth nitrate standard curve.

Methyllycaconitine (MLA) HPLC-MS Sample Preparation and Analysis Assay aliquots were prepared for HPLC-MS analysis as described by Lee and colleagues (2020). Aliquots were centrifuged at 14800 x g for 10 minutes), the supernatant transferred to a new microcentrifuge tube and centrifuged again (14800 x g for 20 minutes). The supernatant (50 µL) was diluted 50:50 with acetonitrile. An aliquot (100 µL) of the dilute assay sample was transferred to a 300 µL autosampler vial for HPLC-MS.

Methyllycaconitine analysis by HPLC was performed following the protocol outlined by Lee and colleagues (2020) with a few modifications. 5 uL samples were injected onto an Acquity UPLC® HSS T3 column (100 x 2.1 mm). Alkaloids were eluted with a gradient flow consisting of 5mM ammonium formate, 0.1% formic acid and acetonitrile at a flow rate of 0.500 mL/min. The mobile phase consisted of 97:3, v:v of 5mM ammonium formate and 0.1% formic acid-acetonitrile for 1 minute followed by a linear gradient to a composition of 50% acetonitrile. After 10 minutes the mobile phase was injected using an Agilent 1290 UHPLC (Thermo Scientific, San

Jose, CA, USA) with the column eluent connected to an Agilent 6538 Q-TOF (Thermo Scientific, San Jose, CA, USA). With this method, methyllycaconitine (MLA) eluted at 7.6 minutes Chromatographic peaks were analyzed by generating reconstructed HLPC-QTOF chromatograms with the calculated MH^+ calculated weight for MLA (MH^+ = 683.3538) with a mass tolerance of 20 ppm.

Data for both total alkaloid and MLA assays were pre-processed in the same manner. To quantify the proportion of total alkaloid or MLA degradation attributable to bacterial (Total degradation – Abiotic activity – (Fungal – Abiotic activities)), fungal (Fungal activity – Abiotic activity), and Abiotic activity, values were adjusted to percent degradation within each assay and each successive proportion of degradation was subtracted from the former. Abiotic proportions were adjusted to a value of zero in the few cases where the separately measured and calculated bacterial and fungal activities were greater than the total (bacterial + fungal + abiotic) degradation observed.

Statistical Analysis Final data for MLA degradation included assays from rumen samples of 10 individuals from each species, excluding elk with just eight (n=68). Data for total alkaloid degradation included assays from rumen samples of 10 individuals from each species, excluding elk and moose with just eight (n=66). Two moose samples were excluded from the analysis due to zero contribution of degradation within each assay. To assess degradation influenced by abiotic, bacterial, or fungal factors (type) simple linear regression was performed on either the proportion of total alkaloid degradation or MLA specifically by type (**Models 1,2**). Additional models were fit to include a species-by-type interaction term to determine whether total- or MLA- degradation influenced by type varied by species (**Models 3,4**). Analyses were conducted in R Statistical

Software. Simple linear models were conducted using the *lm* function in base R Statistical Software. Statistical significance was defined as a *P* value <0.05.

Fungal Isolate Assays

We used fifteen anaerobic fungal isolates routinely cultured in the Elshahed and Youssef laboratory at Oklahoma State University to assay for their individual ability to degrade MLA alkaloids over 60 h in *in vitro* incubations. Fungal isolates were representative of 10 fungal genera, and 9 herbivorous hosts including American bison (*Bison* spp.), wild sheep (*Ovis* spp.), Axis deer (*Axis* spp.), blackbuck deer (*Antilope* spp.), donkey (*Equus* spp.), tunis sheep (*Ovis* spp.), white-tailed deer (*Odocoileus* spp.), and zebra (*Equus* spp.) (**Table 1**). Individual cultures were grown in 9 mL of rumen-fluid (RF) medium with 0.5% cellobiose used as a substrate (Calkins et al., 2016). Cultures were diluted to a concentration of 0.002 ng/mL of MLA from pure extract (Tocris Biosciences, Minneapolis, USA) and inoculated with an antibiotic solution (50 µg/mL kanamycin, 50 µg/mL penicillin, 20 µg/mL streptomycin, and 50 µg/mL chloramphenicol) to inhibit growth of both bacteria and methanogenic archaea (Hanafy et al. 2020). Prior to and following incubations, MLA was extracted and measured using the same HPLC-MS methods as previously described.

Fungal Isolate Statistical Analysis We performed linear mixed-effects regression on the log-transformed MLA concentrations at time 0 and time 48 with a random intercept for ID to account for repeated measures (**Model 5**). Additional models were fit to include an isolate-by-time, species-by-time, and genera-by-time interaction to assess whether an association between MLA degradation and any of these additional variables occurred (**Models 6,7,8**, respectively). For all models the reference for time was 0.

All analyses were conducted in R Statistical Software (version 1.4). Linear models were conducted using the *lmer* function and *lme4* package in R Statistical Software. P values were approximated using Satterthwaite approximation for degrees of freedom in the *afex* package. Statistical significance was defined as confidence intervals excluding 1 and *P* values <0.05.

RESULTS

Total Abiotic, Bacterial, and Fungal Degradation

Degradation of total larkspur alkaloids was predominantly attributable to fungal activity with an average of 67.7 ± standard deviation (SD) of 38% of degradation occurring across all samples (**Figure 1**). Fungal degradation of total alkaloids was significantly higher than both bacterial and abiotic mediated degradation ($P<0.001$) across all samples. However, overall bacterial activity accounted for 25.0 ± SD 33% of degradation of total larkspur alkaloids and was significantly higher than abiotic degradation ($P<0.001$). Abiotic factors did not appreciably contribute to total alkaloid degradation 7.3% ± SD 11% (**Figure 1**). Similarly, MLA-specific degradation was mostly attributable to fungal activity with 55.2 ± SD 36% of MLA-specific degradation occurring in fungal-specific assays. Surprisingly, a significantly higher abiotic effect compared to bacterial degradation ($P<0.001$) was observed that contributed 32.7 ± 30% of the degradation while no significant MLA-specific degradation activity was attributable to bacteria (12.1%).

Abiotic, Bacterial, and Fungal Degradation Contribution Within Species

Microbially mediated degradation of both total alkaloid and MLA also varied by species. In total alkaloid degradation assays, the contribution of fungal mediated activity on degradation

was significantly higher than both abiotic and bacterial degradation for moose ($P<0.001$), mountain goat ($P<0.001$), mule deer ($P<0.01$), pronghorn ($P<0.001$), and white-tailed deer ($P<0.001$) (**Figure 2**). Fungal degradation within the elk species was significantly higher than abiotic ($P<0.001$), but not larger than the percentage of bacterial degradation ($P= 0.1$). In bighorn sheep samples, contribution to total alkaloid degradation bacterial degradation was significantly higher than antibiotic ($P= 0.007$), but not significantly larger than the percentage of fungal degradation ($P= 0.3$).

For MLA specific degradation, the percent degradation due to fungal activity was significantly higher than both abiotic and bacterial in bighorn sheep ($P<0.001$), mountain goat ($P= 0.03$), mule deer ($P<0.001$), and marginally higher than abiotic in pronghorn ($P= 0.05$) (**Figure 3**). In white-tailed deer and elk samples, abiotic and fungal influenced degradation of MLA was significantly higher than bacterial ($P=0.04$, and $P<0.001$, respectively). Abiotic degradation was significantly higher than both bacterial ($P= 0.02$) and fungal ($P= 0.01$) in moose samples.

MLA Degradation by Fungal Isolates

Across the fifteen individual fungal isolates, significant MLA degradation occurred at an average of 31% ($P< 0.001$, 95% CI: 22–38%). Six fungal strains representative of five fungal genera and five wild ruminant hosts showed significant degradative abilities (**Figure 4**). Fungal strain R5, isolated from a wild sheep, demonstrated the highest MLA degradation at 71% ($P<0.001$, 95% CI: 63–77%). Fungal strains ORC_32 and TB_34, isolated from oryx and tunis sheep species degraded MLA at an average of 62% ($P<0.001$, 95% CI: 52–70%), and 52% ($P< 0.001$, 95% CI: 39–62%). ORC_37, AXB_13, isolated from oryx, and axis deer, degraded on average 43% ($P=0.002$, 95% CI: 28–56%) and 43% ($P= 0.002$, 95% CI: 27–56%) of MLA. Fungal

isolate ABS_23, sampled from an American bison degraded an average of 33% of MLA (P= 0.02, 95% CI: 14–47%). The nine other fungal strains did not show demonstrate significant MLA alkaloid degradation.

DISCUSSION

Our results indicate that wild ruminants variably possess a microbial metabolism that can degrade the toxic alkaloids in *Delphinium occidentale* species and that such degradation may be attributed to fungal-mediated activity. Fungal activity was found to have the largest influence on both total alkaloid and MLA specific degradation across the total sample size, as well as within each species. However, abiotic degradation was found to be significant within the MLA specific assays. Rumen bacterial activity did not prove to aid significantly in any alkaloid degradation.

Anaerobic fungi, while low in abundance within the rumen, are extremely important to ruminal fiber degradation. Most of the major polysaccharides of plants are fermented by rumen fungi (Hobson and Stewart, 1997). Using mechanical force, enzymatic reactions, or a combination of both, rumen fungi can penetrate plant cell walls and further break down difficult plant polysaccharides (Kolattukudy, 1985). From the high percentage of fungal mediated alkaloid degradation seen in both total alkaloid and MLA assays, it is possible to assume that ruminal fungi in wild ruminants possess the ability to degrade toxic alkaloids while simultaneously breaking down larger plant matter.

The large percentage of abiotic degradation in MLA specific assays may be a result instability of the purified alkaloid. Information concerning stability of pure Methyllycaconitine citrate, particularly in solution, has seldom been described; however, advice provided by the manufacturer (Tocris Biosciences, Minneapolis, USA) suggests MLA citrate to be stable only at -

20°C. Additionally, MLA is thought to remain stable at neutral or acidic pH (Majak, 1993). Assays were conducted at 39°C with an initial pH at 6.8 to simulate conditions like the ruminal environment. Under such circumstances it is possible that the prolonged exposure to heat may have influenced the instability of the purified Methyllycaconitine alkaloid and the large proportion of degradation in those assays. However, the small proportion of abiotic degradation of total alkaloids from *Delphinium* spp. extract is supported by previous work demonstrating the stability of plant derived alkaloids in rumen fluid (Majak, 1993).

Among the available anaerobic fungal isolates there was consistent MLA degradation that matched the percent of degradation as seen within the wild ruminant samples collected in Montana. Additionally, there were instances of significant degradation by individual species strain, fungal genera, or specific herbivorous host.

Most of the fungal isolates were isolated from either wild or domesticated ruminants, however, two isolates that did not exhibit significant MLA degradation, were isolated from hindgut fermenter herbivores (*Equus* spp.). Host phylogeny, determined by factors such as digestion type, has been shown to potentially determine microbial composition in herbivorous hosts with animals of similar digestion generally sharing similar communities (O' Donnell et al., 2017). Additionally, *Equus* spp. are considered to follow a grazer type feeding niche and are not likely to be exposed to high levels of secondary plant compounds (Palo and Robbins, 1991). Non-ruminants may not possess gut microorganisms capable of degrading toxic secondary plant compounds such as MLA due to difference in exposure and/or microbial community composition.

The remaining isolates were cultured from either domesticated or wild ruminants. Isolates with the highest degradation of MLA were isolated from multiple wild ruminant species including

American bison, wild sheep, axis deer, oryx, and tunis sheep. In these instances, the animal host was likely exposed to either Delphinium species containing some level of MLA concentration, or plants with similar secondary plant compound composition. Fungal isolates from domesticated ruminants did not exhibit significant MLA degradation suggesting these animals likely were not exposed to plants containing similar secondary plant compounds to that of tall larkspur.

Ruminants are unique in the fact that they can acquire tolerance to increased exposure of toxic secondary plant compounds. Often this ability may be contributed to alterations in populations of rumen microbes (Hobson and Stewart, 1997). Management practices that take advantage of adapted rumen microbiota may be useful in preventing further toxicity problems in livestock. A potential management option for larkspur toxicosis in Montana rangeland cattle may be to isolate and culture fungal strains from local ruminants' species and test for their individual abilities to degrade both total alkaloids from *Delphinium* spp. and methyllycaconitine specifically. High alkaloid degrading fungi may then be considered for ruminal inoculation into livestock in hopes of establishing a ruminal population capable of toxic metabolite degradation. Similar microbial inoculation has been shown to successfully alter cattle rumen microbiome to aid in both toxic compound degradation (Hammond, 1995) and enhanced feed digestion (Ribeiro et al., 2017).

Minor limitations were apparent during the development of this study. Because samples were collected in the field by non-trained volunteers, it is possible there was loss in viability due to both exposure to oxygen outside of the rumen or contamination during collection. It is nearly impossible to avoid these concerns when collecting samples from wild animals outside of a laboratory. However, our results show significant differences in degradation based on abiotic,

bacterial, and fungal activity, indicating our ability to successfully culture rumen microbiota capable of toxic alkaloid degradation.

These data provide promising potential for future use of microbial isolates as direct fed microbial supplements for rangeland cattle to lessen the risk of larkspur toxicosis. Significant fungal-mediated alkaloid degradation within Montana's wild ruminant species as well as the high degradation demonstrated by the available fungal isolates demonstrates the possible use of transplantation of these microbes into ruminant's incapable of larkspur alkaloid degradation. Use of such oral supplements would be of substantial benefit for rangeland cattle to alleviate the negative effects associated with larkspur toxicosis.

TABLES AND FIGURES

Table 1. Table includes individual fungal isolate identification (ID) and their associated genera and herbivorous host in which they were isolated from.

Isolate ID	Genera	Herbivorous Host
ABC_22	*Orpinomyces*	American bison
ABS_23	*Anaeromyces*	American bison
AS_32	*Pecoramyces*	Wild sheep
AXB_13	*Piromyces sp. A*	Axis deer
BB_2	*Paucimyces*	Blackbuck deer
Cap_2a	*Capellomyces*	Wild sheep
DonB_21	*Piromyces finnis*	Donkey
ORC_32	*Pecoramyces*	Oryx
ORC_37	*Liebetanzomyces*	Oryx
ORS_33	*Piromyces*	Oryx
R5	*Aestipasuomyces*	Wild sheep
TB_34	*Undetermined*	Tunis sheep
TS_14	*Undetermined*	Tunis sheep
WTS_53	*Akliashbomyces*	White-tailed deer
Zn_4	*Khyollomyces*	Zebra

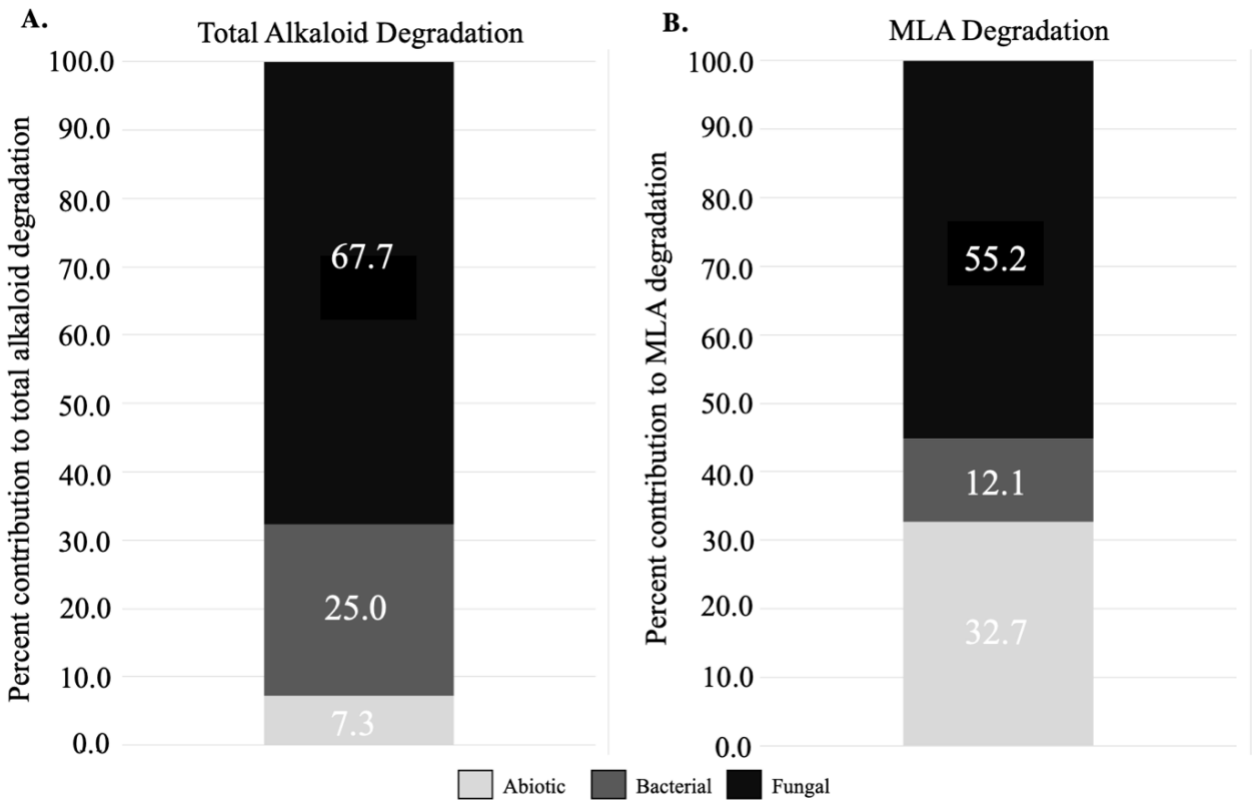


Figure 1. Percent alkaloid degradation by type. Percentage of total alkaloid (A) and Methyllycaconitine (B) degradation influenced by abiotic bacterial, and fungal activity.

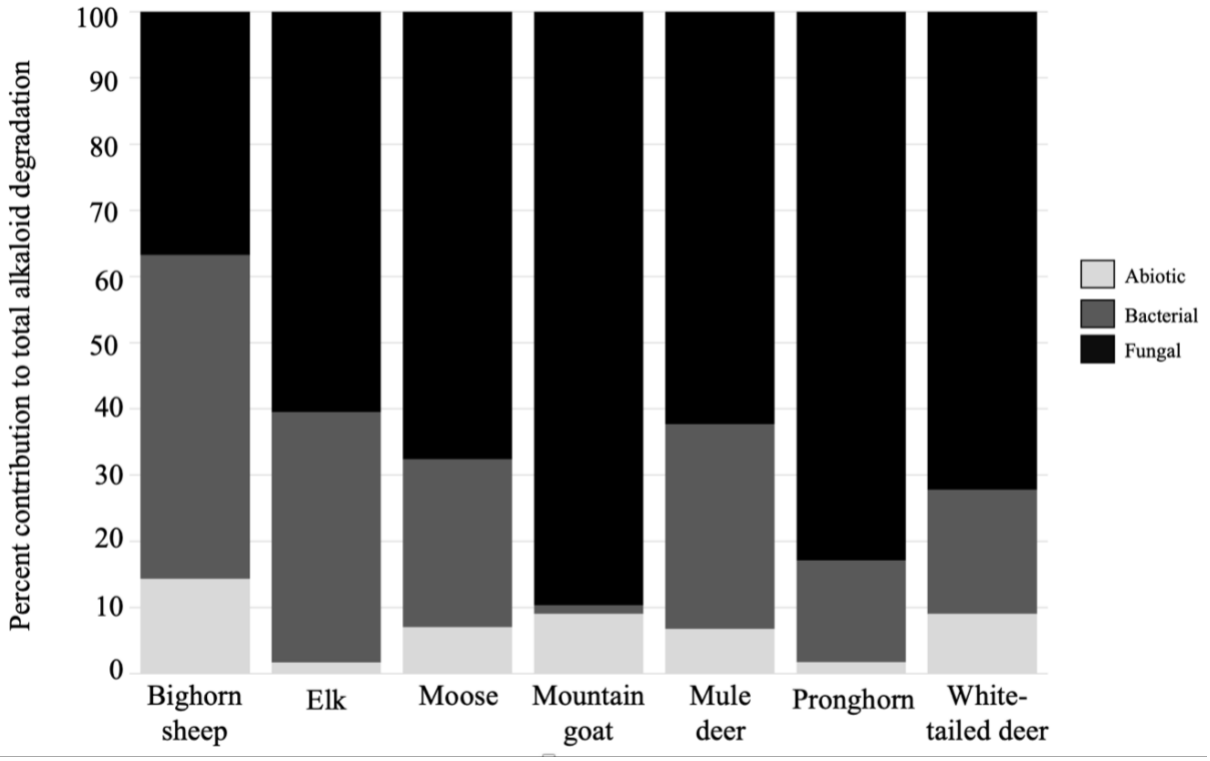


Figure 2. Percent total alkaloid degradation by species. Percentage of total alkaloid degradation influenced by abiotic, bacterial, and fungal activity within each species.

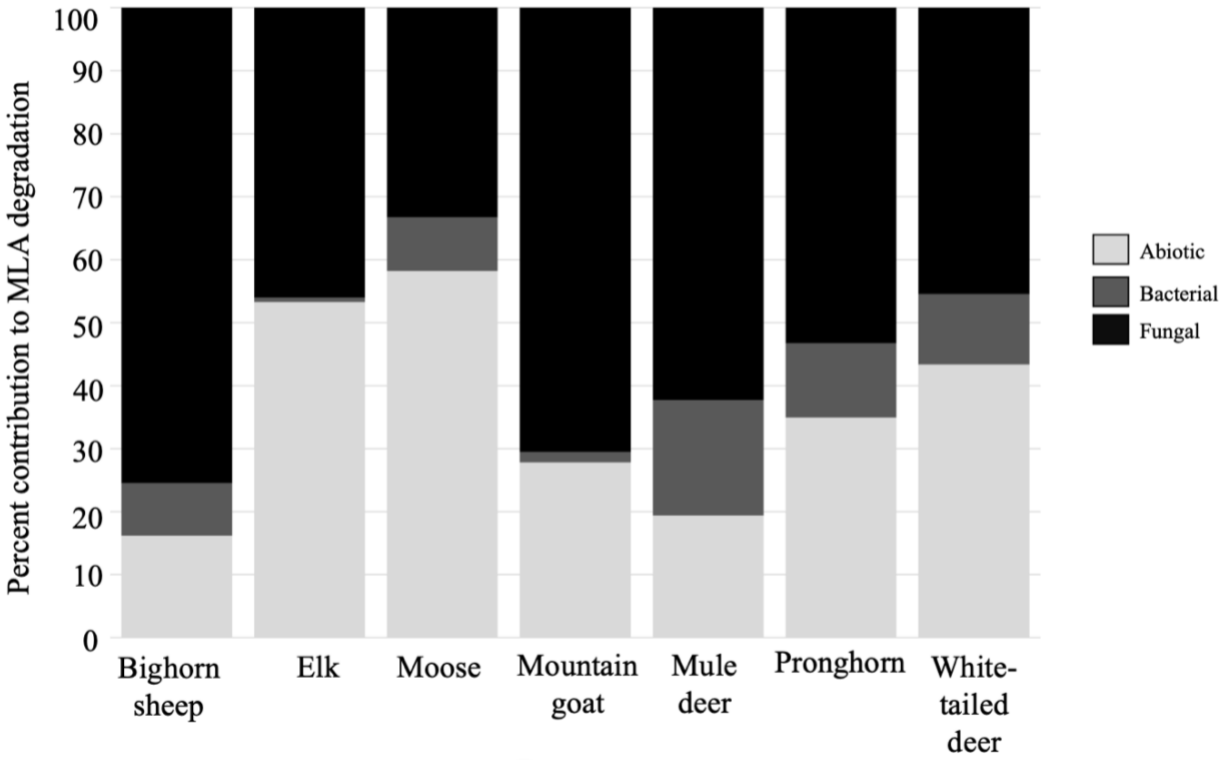


Figure 3. Percent MLA degradation by species. Percentage of MLA degradation influenced by abiotic, bacterial, and fungal activity within each species.

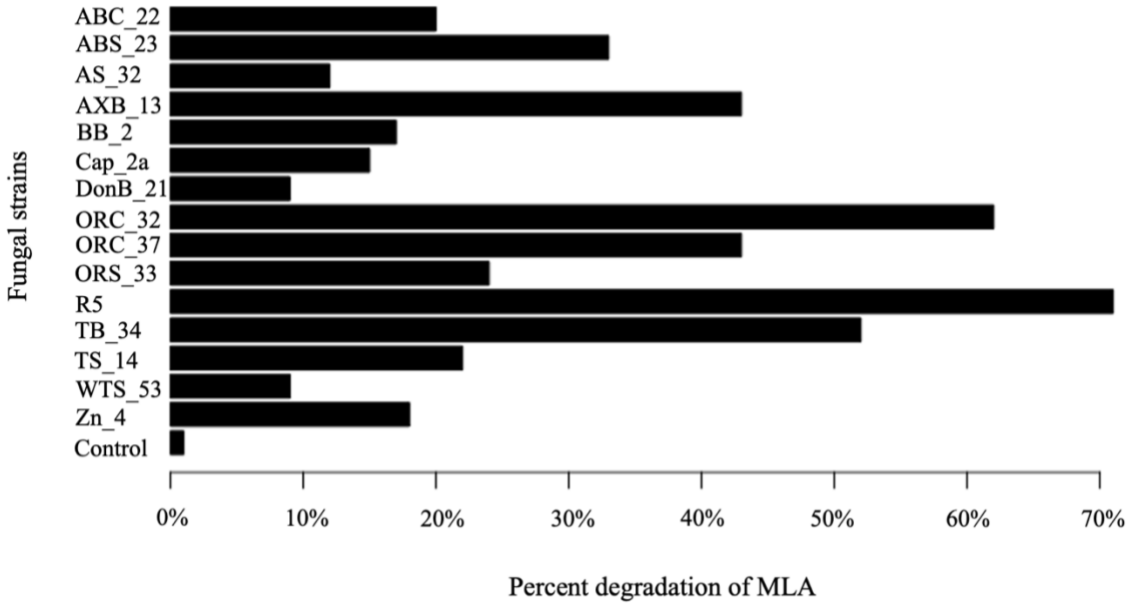
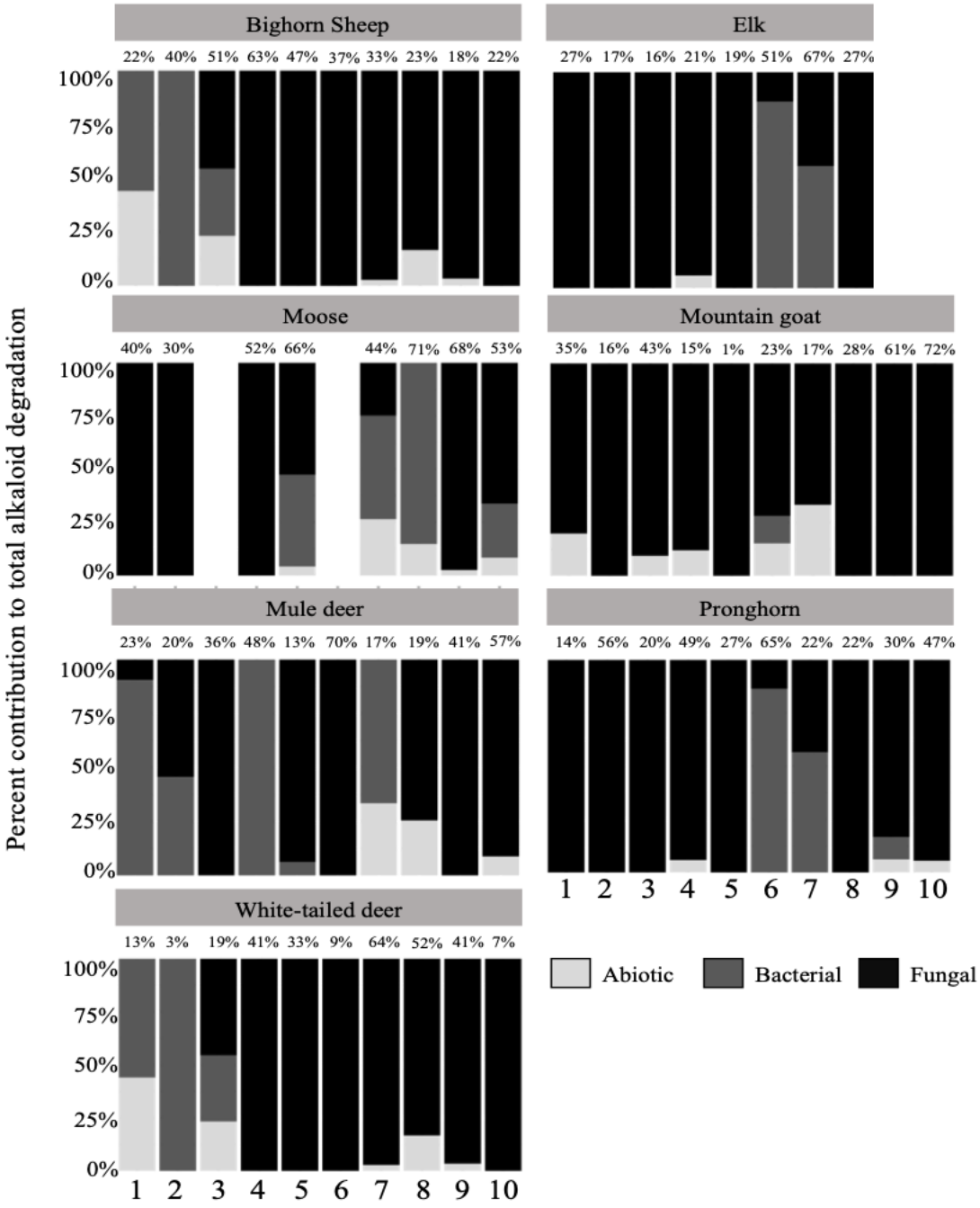
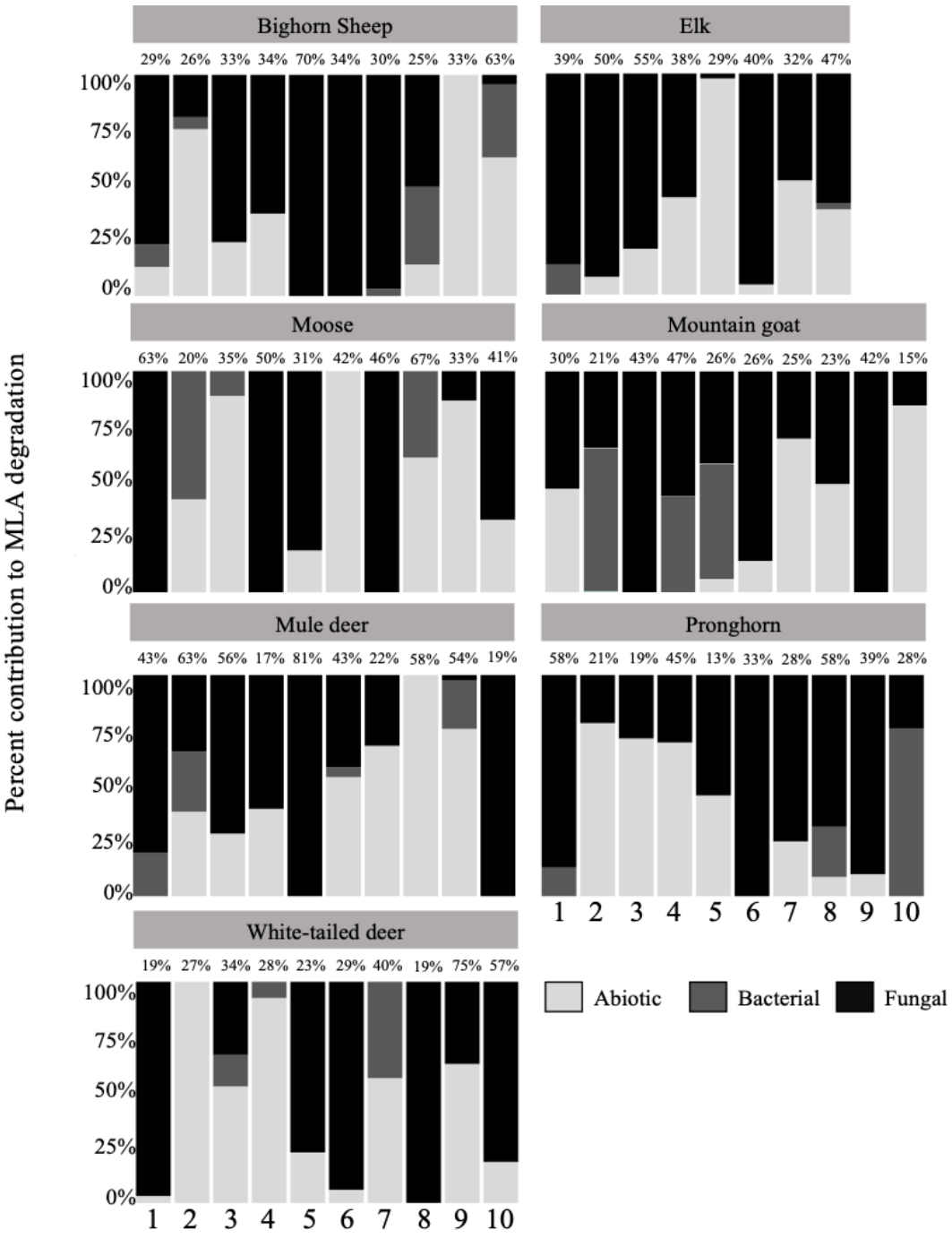


Figure 4. Percent degradation of MLA by individual fungal strains. Percentage of MLA degradation influenced the activity of fungal strains isolated from the gastrointestinal tract of various wild herbivores.

SUPPLEMENTAL MATERIAL



Supplemental Figure 1. Percent total alkaloid degradation by individual. Percent contribution of abiotic, bacterial, and fungal degradative activity on total alkaloid degradation by individual within each species. precents above each bar illustrate the total percent degradation within each individual.



Supplemental Figure 2. Percent MLA degradation by individual. Percent contribution of abiotic, bacterial, and fungal degradative activity on MLA degradation by individual within each species. Moose ID 2 and 5 exhibited no MLA degradation. precents above each bar illustrate the total percent degradation within each individual.

References Cited

Aiyar, V.N., M.H. Benn, T. Hanna, J. Jacyno, S.H. Roth, and J.L. Wilkens. 1979. The principal toxin of Delphinium brownie Rybd., and its mode of action. Experientia, 35, 1367.

Aldous, A.E., 1917. Eradicating tall larkspur on cattle ranges in national forests. USDA Farmers Bull. 826, Washington, DC.

Aldous, A.E., 1917. Eradicating tall larkspur on cattle ranges in national forests. USDA Farmers Bull. 826, Washington, DC.

Benn, M.H., and J.M. Jacyno. 1983. The toxicology and pharmacology of the diterpenoid alkaloids in Alkaloids: Chemical and Biological Perspectives. Pelletier, S.W., ed Wiley, New York, pp. 153-210.

Calkins, S. N.C. Elledge, R.A. Hanafy, M.S. Elshahed, N. Youssef. A fast and reliable procedure for spore collection from anaerobic fungi: Application for RNA uptake and long-term storage of isolates. J. Microbiobiological Methods. 127: 206-213.

Dobelis, P., J.E. Madl, J.A. Pfister, G.D. Manners, and J.P. Walrond. 1999. Effects of Delphinium Alkaloids on Neuromuscular Transmission. The journal of Pharmacology and Experimental Therapeutics. 291(2):538-546.

Holechek, J.L. 2002. Do most livestock losses to poisonous plants result from "poor" range management? J. Range Management 55:270-276.

Kolattukudy, P.E. 1985. Enzymatic Penetration of the Plant Cuticle by Fungal Pathogens. Ann. Rev. Phytopatology. 23: 223-250.

Lee, S.T., K.D. Welch, C.A. Stonecipher, D. Cook, D.R. Gardner, and J.A. Pfister. 2020. Analysis of rumen content and ocular fluid for toxic alkaloids from goats and cows dosed larkspur (*Delphinium barbeyi*), lupine (*Lupinus leucophyllus*), and death camas (*Zigadenus paniculatus*). Toxicon. 176:21-29.

Loh, Z.H. D. Ouwerkerk, A.V. Klieve, N.L. Hungerford, and M.T. Fletcher. 2020. Toxin Degradation by Rumen Microorganisms: A Review. Toxins. 12,664; doi:10.3390/toxins12100664

Nielsen, D.B., and M.H. Ralphs. 1987. Larkspur economic considerations. In: James, L.F., Ralphs, M.H., Nielsen, D.B. (Eds.), Ecology and Economic Considerations of Poisonous Plants. Westview Press, Boulder, *CO*, pp. 119–129.

Olsen, J.D. 1978. Larkspur Toxicosis: A Review of Current Research. Academic Press, pp. 535-543.

O'Donnell, H.M.B. Harris, R.P. Ross, and P.W. O'Toole. 2017. Core fecal microbiota of domesticate herbivorous ruminants, hindgut fermenters, and monogastric animals. Microbiologyopen. 6(5): :e00509. doi: 10.1002/mbo3.509.

Palo, R.T., and C.T. Robbins. 1991. Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton, FL. Pp. 103-115.

Panter, K.E., G.D. Manners, B.L. Stegelmeier, S. Lee, D.R. Gardner, M.H. Ralphs, J.A. Pfister, and L.F. James. 2002. Larkspur poisoning: toxicology and alkaloid structure-activity relationships. Biochemical Systematics and Ecology. 30(2):113-128.

Pfister, J.A., G.D. Manners, D.R. Gardner, K.W. Price, and M.H. Ralphs. 1996. Influence of alkaloid concentration on acceptability of tall larkspur (*Delphinium* spp.) to cattle and sheep. Journal of Chemical Ecology. 22(6):1147-1148.

Ribeiro, G.O., D.B. Oss, Z. He, R.J. Gruninger, C. Elekwachi, R.J. Froster, W. Yang, K.A. Beauchemin, and T.A. McAllister. Repeated inoculation of cattle rumen with bison rumen contents alters the rumen microbiome and improves nitrogen digestibility in cattle. Scientific Reports. 7:1276. DOI:10.1038/s41598-017-01269-3

Sreevidya, N., S. Mehrotra. 2003. Spectrophotometric Method for Estimation of Alkaloids Precipitable with Dragendorff's Reagent in Plant Materials. Journal of AOAC International. 86(6):1124-1127.

Welch, K.D., D.R. Gardner, B.T. Green, C.A. Stonecipher, D. Cook, J.A. Pfister. 2006. Comparison of the serum toxicokinetics of larkspur toxins in cattle, sheep, and goats. Toxicon. 119:270-273.

Welch, K.D., B.T. Green, D.R. Gardner, D. Cook, and J.A. Pfister. 2015. The effect of administering multiple doses of tall larkspur (*Delphinium barbeyi*) to cattle. 93:4181-4188.

CHAPTER FIVE

CONCLUSION

Our results indicate a rumen-located, microbial metabolism exists within Montana's wild ruminants, that can degrade the toxic alkaloids found in *Delphinium occidentale* species, including the highly toxic MLA. Various plant species have adapted the ability to produce toxic alkaloids as means for protection (Palo and Robbin, 1991). In mammalian herbivores, selective pressures for adapting the ability to successfully digest secondary plant compounds are highly influenced by exposure to such compounds through consumption. Therefore, it is likely that feeding niches play a role in determining an individual's ability to mitigate toxicity. Browsers, including most of the wild ruminants found in Montana, whose diets consists of forbs and shrubs/trees, are likely to consume higher levels of secondary plant compounds than that of grazers (cattle) whose diets are often restricted to grasses and leaf tissue (Palo and Robbins, 1991). The results from our initial assays (chapter 2) are consistent with these indications as each of the wild ruminant species variably demonstrated the ability to successfully degrade alkaloids found in the *Delphinium* plant species. While no evidence has been presented to indicate wild ruminants are feeding on larkspur specifically, it is reasonable to assume that they are ingesting plants with similar secondary plant compound constitution and have acquired the ability to successfully metabolize such structures.

Further, wild ruminants in Montana have shown to variably possess a microbial metabolism capable of toxic alkaloid degradation. Degradation of total larkspur alkaloids and MLA was predominantly influenced by fungal-mediated activity. Anaerobic fungi within the rumen play a large role in ruminal fiber degradation as they can use enzymatic and mechanical force to penetrate plant cell walls and break down difficult plant polysaccharides (Kolattukudy,

1985). It may be likely that while breaking down larger plant matter, ruminal fungi are capable of simultaneously degrading alkaloid compounds.

Among the anaerobic fungal isolates tested, MLA degradation occurred at a significant percentage consistent with that of degradation seen within the wild ruminant samples collected in Montana. The fungal isolates that demonstrated the most MLA degradation were sampled from various wild ruminant species that were likely exposed to *Delphinium* plants specifically during forage, or plant species with similar secondary plant compound composition. Additionally, fungal isolates sampled from non-ruminant hindgut herbivores were incapable of significant alkaloid degradation suggesting alkaloid degradation may be attributed to the mechanisms utilized by anaerobic rumen fungi.

Several minor limitations were apparent in each study. Samples were collected in the field by hunters during dressing of their wild game harvest. While hunters were briefly trained in sterile technique and provided with written and visual instructions to obtain the rumen samples, collections were not monitored by trained members of the laboratory so neither sterility nor accurate rumen capture can be guaranteed. Depending on the size of the animal and location of harvest, time from sample collection to sample freezing could have, in some cases, taken anywhere from several hours to days. Additionally, rumen microbes are sensitive to oxygen and were exposed to some degree during sample collection; it is possible some viability of organisms was lost due to this unavoidable step. Despite these limitations, evidence indicated viable microorganisms were successfully captured from the wild ruminant samples and demonstrated their ability to degrade toxic plant alkaloids found in the *Delphinium* spp.

These data provide the solid foundation for future studies. While we know the percent of fungal-mediated alkaloid degradation in wild ruminant samples collected in Montana were significantly high, we have not yet attempted to isolate fungal species from these samples. The interest in microbially mediated degradation of the toxic alkaloids in larkspur plants and the ability to isolate the microorganisms influencing such degradation would be of great benefit for livestock producers. The potential to isolate fungal species from wild ruminants and use as a direct fed microbial to livestock to mitigate larkspur toxicosis would be substantial. Further studies should aim to determine the specific specie(s) largely responsible for larkspur alkaloid degradation, specifically MLA and whether the introduction of such fungal specie(s) to cattle would help to alleviate the deleterious effects of larkspur toxicosis.

References Cited

Kolattukudy, P.E. 1985. Enzymatic Penetration of the Plant Cuticle by Fungal Pathogens. Ann. Rev. Phytopathology. 23: 223-250.

Palo, R.T., and C.T. Robbins. 1991. Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton, FL. Pp. 103-115.

REFERENCES CITED

Aiyar, V.N., M.H. Benn, T. Hanna, J. Jacyno, S.H. Roth, and J.L. Wilkens. 1979. The principal toxin of Delphinium brownie Rybd., and its mode of action. Experientia, 35, 1367.

Aldous, A.E., 1917. Eradicating tall larkspur on cattle ranges in national forests. USDA Farmers Bull. 826, Washington, DC.

Aldous, A.E., 1917. Eradicating tall larkspur on cattle ranges in national forests. USDA Farmers Bull. 826, Washington, DC.

Benn, M.H., and J.M. Jacyno. 1983. The toxicology and pharmacology of the diterpenoid alkaloids in Alkaloids: Chemical and Biological Perspectives. Pelletier, S.W., ed Wiley, New York, pp. 153-210.

Benson, A. K., S. A. Kelly, R. Legge, F. Ma, S. J. Low, J. Kim, M. Zhang, P. L. Oh, D. Nehrenberg, K. Hua, S. D. Kachman, E. N. Moriyama, J. Walter, D. A. Peterson, and D. Pomp. 2010. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc. Natl. Acad. Sci. USA 107:18933–18938. http://dx.doi.org/10.1073/pnas.1007028107

Calkins, S. N.C. Elledge, R.A. Hanafy, M.S. Elshahed, N. Youssef. 2012A fast and reliable procedure for spore collection from anaerobic fungi: Application for RNA uptake and long-term storage of isolates. J. Microbiological Methods. 127: 206-213.

Chaucheryras-Durand, F., and F. Ossa. 2014. Review: The rumen microbiome: Composition, abundance, diversity, and new investigative tools. The Professional Animal Scientist. 30:1-12.

Cheeke, P.R. 1998. Natural Toxicants in feeds, forages, and poisonous plants. Interstate Publication Inc., Danville, IL.

Cook, D., B.T. Green, K.D. Welch, D.R. Gardner, J.A. Pfister, and K.E. Panter. Comparison of the toxic effects of two duncecap larkspur (*Delphinium occidentale*) chemotypes in mice and cattle. Am. J. Veterinary Research. 72(5):706-714.

Dobelis, P., J.E. Madl, J.A. Pfister, G.D. Manners, and J.P. Walrond. 1999. Effects of Delphinium Alkaloids on Neuromuscular Transmission. The journal of Pharmacology and Experimental Therapeutics. 291(2):538-546.

Dozortseva-Kubanova, P.M. Farmakol. Toksikol. 1959. *22:* 30.

Fowler, M.E. 1983. Plant Poisoning in Free-living Wild Animals: a Review. J. Wildlife Diseases. 19(1): 34-43.

Green, B.T., K.D. Welch, J.A. Pfister, C.G. Chitko-McKown, D.R. Gardner, and K.E. Panter. 2014. Mitigation of Larkspur Poisoning on Rangelands Through the Selection of Cattle. Rangelands. 36(1): 10-15. doi: 10.2111/RANGELANDS-D-13-00031.1

Green B.T., D.R. Gardner, D. Cook, J.A. Pfister, K.D. Welch, and J.W. Keele. 2018. Age-dependent Intoxication by larkspur (*Delphinium*) In Angus Steers. Toxicon 152: 57-59.

Green, B.T., S.T. Lee, D.R. Gardner, K.D. Welch, and D. Cook. 2019. Bioactive Alkaloids from Plants Poisonous to Livestock in North America. Isr. J. Chem. 59: 351-359.

Hammond, A. C. 1995. Leucaena toxicosis and Its control in ruminants. *J. Anim. Sci.*, *73*: 1487-1492.

Heaton, M.P., C.G., Chitko-McKnown, W.M. Grosse, J.W. Keele, J.E. Keen, and W.W. Laergreid. 2001. Interleukin-8 haplotype structure from nucleotide sequence variation in commercial populations of U.S. beef cattle. Mamm. Genome 12: 219-226.

Hobson, P.N., and C.S. Stewart. 1977. The Rumen Microbial Ecosystem. Chapman & Hall, New York, 719pp.

Holechek, J.L. 2002. Do most livestock losses to poisonous plants result from "poor" range management? J. Range Management 55:270-276.

Holloway, M.G. E.V. Laz, and D.J. Waxman. 2006. Codependence of growth hormone-responsive, sexually dimorphic hepatic gene expression on signal transducer and activator of transcription 5b and hepatic nuclear factor 4alpha. Mol. Endocrinol. 20:647-660

James, L.F., D.B. Nielsen, and K.E. Panter. 1992. Impact of poisonous plants on the livestock industry. J. Range Management. 45:3-8.

Kamra, D.N. 2005. Rumen Microbial Ecosystem. Current Scicnce. 89(1): 124-135.

Knight, A. P., and J.A. Pfister. 1997. Larkspur poisoning in livestock: Myths and misconceptions. *Society for Range Management*, *19*(3): 10-13.

Kolattukudy, P.E. 1985. Enzymatic Penetration of the Plant Cuticle by Fungal Pathogens. Ann. Rev. Phytopathology. 23: 223-250.

Lee, S.T., K.D. Welch, C.A. Stonecipher, D. Cook, D.R. Gardner, and J.A. Pfister. 2020. Analysis of rumen content and ocular fluid for toxic alkaloids from goats and cows dosed larkspur (*Delphinium barbeyi*), lupine (*Lupinus leucophyllus*), and death camas (*Zigadenus paniculatus*). Toxicon. 176:21-29.

Loh, Z.H. D. Ouwerkerk, A.V. Klieve, N.L. Hungerford, and M.T. Fletcher. 2020. Toxin Degradation by Rumen Microorganisms: A Review. Toxins. 12,664; doi:10.3390/toxins12100664

Mabjeesh SJ, Cohen M, Arieli A. 2000. In Vitro Methods for Measuring the Dry Matter Digestibility of Ruminant Feedstuffs: Comparison of Methods and Inoculum Source J. Dairy Sci. 83: 2289 - 2294

Majak, W. 1993. Alkaloid levels in a species of low larkspur and their stability in rumen fluid. Society for Range Management. 46(2): 100-103.

Matsuura HN, Rau MR, Fett-Neto AG. 2014.Oxidative stress and production of bioactive monoterpene indole alkaloids: biotechnological implications. Biotechnol Lett. 36:191–200.

McDougall EI. 1948. Studies on ruminant saliva. I. The composition and output of sheep's saliva. Biochem. J. 43: 99 – 109

Miller, A.W., K.D. Kohl, M.D. Dearing. 2014. The gastrointestinal tract of white-throated woodrat (Neotoma albigula) harbors distinct consortia of oxalate-degrading bacteria. Applied and Environmental Biology. 80(5). https://doi.org/10.1128/AEM.03742-13

Nielsen, D.B., and M.H. Ralphs. 1987. Larkspur economic considerations. In: James, L.F., Ralphs, M.H., Nielsen, D.B. (Eds.), Ecology and Economic Considerations of Poisonous Plants. Westview Press, Boulder, *CO*, pp. 119–129.

O'Donnell, H.M.B. Harris, R.P. Ross, and P.W. O'Toole. 2017. Core fecal microbiota of domesticate herbivorous ruminants, hindgut fermenters, and monogastric animals. Microbiologyopen. 6(5): :e00509. doi: 10.1002/mbo3.509.

Olsen, J.D. 1978. Larkspur Toxicosis: A Review of Current Research. Academic Press, pp. 535-543.

Palo, R.T., and C.T. Robbins. 1991. Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton, FL. Pp. 103-115.

Panter, K.E., T. D. Bunch, R. F. Keeler, D. V. Sisson, and R. J. Callan. 1990. J. Toxicol. Clin. Toxicol, 28: 69–83.

Panter, K.E., L. F. James, and D. R. Gardner. 1999. Lupines, poison-hemlock and Nicotiana spp: toxicity and teratogenicity in livestock J. Nat. Toxins, 8: 117–34.

Pfister, J.A., G.D. Manners, M.H. Ralphs, Z.X. Hong, and M.A Lane. 1988. Effects of Phenology, Site, and Rumen fill on Tall Larkspur Consumption by Cattle. J. Range Management. 41(6): 509-514.

Pfister, J.A., G.D. Manners, D.R. Gardner, K.W. Price, and M.H. Ralphs. 1996. Influence of alkaloid concentration on acceptability of tall larkspur (*Delphinium* spp.) to cattle and sheep. Journal of Chemical Ecology. 22(6):1147-1148.

Panter, K.E., G.D. Manners, B.L. Stegelmeier, S. Lee, D.R. Gardner, M.H. Ralphs, J.A. Pfister, and L.F. James. 2002. Larkspur poisoning: toxicology and alkaloid structure-activity relationships. Biochemical Systematics and Ecology. 30(2):113-128.

Pfister, J.A., D.R. Gardner, K.E. Panter, G.D. Manners, M.H. Ralphs, B.L. Stegelmeier, and T.K. Schoh. 1999. Larkspur (*Delphinium* spp.) Poisoning in Livestock. J. Nat Toxins 8: 81-94.

Pfister, J.A., and D.R. Gardner, Consumption of low Larkspur (Delphinium nuttallianum) by Cattle. J. Natural Toxins. 8(1): 27-34.

Pfister, J.A. D.R. Gardner, and E.A. Burritt. 2014. Grazing Tall Larkspur Ranges: A livestock Producer's Decision-making Handbook. Environmental Science.

Ralphs, M. H., J. A. Pfister, and J. D. Olsen. 1988. Reducing larkspur poisoning in cattle on mountain ranges. Utah Science. 50(2): 109-115.

Ribeiro, G.O., D.B. Oss, Z. He, R.J. Gruninger, C. Elekwachi, R.J. Froster, W. Yang, K.A. Beauchemin, and T.A. McAllister. Repeated inoculation of cattle rumen with bison rumen contents alters the rumen microbiome and improves nitrogen digestibility in cattle. Scientific Reports. 7:1276. DOI:10.1038/s41598-017-01269-3

Shipley, L.A. 2001. Grazers and Browsers: How Digestive Morphology Affects Diet Selection.

Sreevidya, N., S. Mehrotra. 2003. Spectrophotometric Method for Estimation of Alkaloids Precipitable with Dragendorff's Reagent in Plant Materials. Journal of AOAC International. 86(6):1124-1127.

Uyeno, Y., Y. Sekiguchi, K. Tajima, A. Tak-enaka, M. Kurihara, and Y. Kamagata. 2010. An rRNA-based analysis for evaluating the effect of heat stress on the rumen microbial composition of Holstein heifers. Anaerobe 16:27–33. http://dx.doi.org/10.1016/j.anaer obe.2009.04.006.

Vilariño M.P., and D.A Ravetta. 2008. Tolerance to herbivory in lupin genotypes with different alkaloid concentration: interspecific differences between Lupinus albus L. and L. angustifolius L. Environ Exp Bot. 63:130–6.

Welch, K.D., B.T. Green, D.R. Gardner, D. Cook, and J.A. Pfister. 2015. The effect of administering multiple doses of tall larkspur (*Delphinium barbeyi*) to cattle. 93:4181-4188.

Welch, K.D., D.R. Gardner, B.T. Green, C.A. Stonecipher, D. Cook, J.A. Pfister. 2006. Comparison of the serum toxicokinetics of larkspur toxins in cattle, sheep, and goats. Toxicon. 119:270-273.