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Understanding Plant Secondary Metabolites; Above and Below Ground

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UNDERSTANDING PLANT SECONDARY METABOLITES;
ABOVE AND BELOW GROUND

by

Andrea K. Clemensen

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

of

DOCTOR OF PHILOSOPHY

in

Ecology - Wildland Resources

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2018
ABSTRACT

Understanding Plant Secondary Metabolites; Above and Below Ground

by

Andrea K. Clemensen, Doctor of Philosophy
Utah State University, 2018

Major Professor: Dr. Juan J. Villalba
Department: Wildland Resources

Plant secondary metabolites (PSM) may increase the sustainability of agroecosystems by reducing inputs and slowing nitrogen mineralization, as PSM act as pesticides and insecticides in plants, anthelmintics in grazing animals, and influence soil nutrient cycling. Thus, it is important to understand what affects PSM in plants, and how PSM influence soil dynamics. Theories suggest that incorporating alfalfa (*Medicago sativa* L.; Alf) or birdsfoot trefoil (*Lotus corniculatus* L.; BFT) into endophyte-infected tall fescue (*Festuca arundinacea* Schreb.; E+TF) pasturelands may improve livestock production. I investigated how planting configuration might influence PSM and nitrogen concentration in these forages. Total nitrogen, and condensed tannins (CT), saponins, and ergovaline (EV) were compared in BFT, Alf, and E+TF, respectively, when forages grew in monocultures and all possible two-way mixtures (Mix). Ergovaline (*p* < 0.01) and N (*p* < 0.001) in E+TF was greater when growing adjacent to legumes than in monoculture,
and N and EV in E+TF were positively correlated ($r = 0.51; p = 0.001$). An *in vitro* trial determined that EV bound with saponins more readily (39%; SE = 0.25) than CT (5.1%; SE = 5.13; $p < 0.05$). I investigated how different N sources, fecal manure and synthetic N fertilizer (urea), affect saponins, CT, EV, and gramine in Alf, BFT, E+TF, and reed canarygrass (*Phalaris arundinacea* L.; RCG), respectively. Total N in E+TF and RCG was greater ($p < 0.05$) in urea- than unfertilized-plots. Seasonal variation in PSM and N was significant ($p < 0.003$) across all species and was species-specific. I investigated whether cattle-grazed pasture forages Alf or sainfoin (*Onobrychis viciifolia* Scop.) in monocultures and Mix with E+TF produce sufficient amounts of saponins or CT, respectively, to effect soil nutrient cycling. I found greater ($p = 0.02$) soil nitrate in Alf than sainfoin plots, yet total N differences between legumes were minimal. I observed greater soil microbial respiration ($p = 0.005$) and dehydrogenase activity ($p = 0.05$) in grass monocultures, indicating both legumes and inherent PSM may inhibit soil microbial and enzyme activity. A laboratory soil-feces incubation study determined cattle feces from differing diets containing CT and saponins decreased mineralization ($p < 0.0001$).
Understanding Plant Secondary Metabolites; Above and Below Ground
Andrea K. Clemensen

Plants naturally produce primary and secondary metabolites. Primary metabolites are directly involved with plant growth and metabolic function. Plant secondary metabolites (PSM) were once thought of as metabolic waste products, and more recently viewed as toxins to herbivores. However, ongoing research shows that PSM are beneficial to herbivores at low doses, and PSM aid plants by attracting pollinators, recovering from injury, protecting from ultraviolet radiation, increasing drought tolerance, and aid in defense against pathogens, diseases, and herbivores. Plant secondary metabolites also influence soil nutrient cycling, and can increase the sustainability of agroecosystems. Endophyte-infected tall fescue (*Festuca arundinacea* Schreb.), which contains ergovaline, and reed canarygrass (*Phalaris arundinacea* L.), containing gramine, were studied along with the legumes alfalfa (*Medicago sativa* L.) which contains saponins, and tannin-containing sainfoin (*Onobrychis vicifolia* Scop.) and birdsfoot trefoil (*Lotus corniculatus* L.). This dissertation researches (i) how planting configuration (monocultures vs. two-way mixtures) influences PSM and total N in plants, (ii) how cattle grazing forages containing PSM affects soil quality, nutrient cycling, and PSM, and (iii) how cattle manure from different diets, containing different PSM, influences soil nutrient cycling.
DEDICATION

This dissertation is dedicated to my friend, advisor, and inspiration, Ron Ryel. Thank you for giving me confidence to dive into the unknown. Also to Bert D. Tanner, whose memories kept me going during times when my heart doubted my competence. See you both on the other side.

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CHAPTER 1
INTRODUCTION

1. Introduction

Since the beginning of the 20th century the population in the United States has more than quadrupled, from 75 million to over 320 million (National Research Council, 2010). Despite this rapid rise, land use for agriculture has remained largely unchanged, while U.S. agricultural exports are greater than agricultural imports (USDA ERS - Agricultural Trade, 2017). This proficiency is possible by the industrialization of conventional agriculture. Conventional crop production makes use of synthetic pesticides, herbicides, and fertilizers whereas conventional animal production may use vaccines, antibiotics, medicated feeds, and growth hormones. The industrialization of conventional agriculture is based on the idea of large-scale specialization of either animal or crop production for mass production (National Research Council, 2010). Such specialization has produced negative environmental impacts which challenge the sustainability of agroecosystems. In turn, several strategies are emerging to attenuate those negative impacts created by large-scale crop production systems.

1.2. Environmental impact

As productive as U.S. farms have become, a growing body of research has confirmed a range of negative impacts on the environment. Conventional agriculture is a major contributor to global greenhouse gases, loss of plant biodiversity and soil organic matter, and has led to the degradation of natural resources, natural water bodies, and
public health (Bauer and Black, 1981; National Research Council, 2010; Nixon, 1995; Vitousek et al., 1997).

The Environmental Protection Agency reported that in 2010 agriculture was responsible for 6.3 percent of the total greenhouse gas emissions in the United States. Greenhouse gas emissions from agricultural use are dominated by nitrous oxide (N₂O) from management activities such as fertilizer use, manure application, and the utilization of nitrogen-fixing crops. Another large contributor to greenhouse gas emissions is by ruminant animals. Enteric fermentation in ruminants emits methane gas (CH₄) into the atmosphere. The agricultural sector is the largest contributor to N₂O and CH₄ emissions, both with higher global warming potential than CO₂ in the United States (EPA, 2017).

The Food and Agriculture Organization (FAO) estimates that 75 percent of agricultural crop biodiversity was lost in the 20th century (FAO, 1998). Loss in agricultural biodiversity has rendered crops susceptible to diseases and pests (Wilkes et al., 1984). Concerns over irreversible genetic loss for plant breeding have led to substantial efforts in protecting and preserving genetic variability (FAO, 2011). Genetic variation is essential to species adaptability through ever-changing environments.

In the central portion of the United States known as the U.S. Corn Belt, the majority of lands have been converted to the production of corn (Liebman et al., 2013), most of which is used as the main energy ingredient for livestock feed. The USDA NRCS (2013) estimated that across the Corn Belt region 9.6 metric tons of soil is lost per ha year⁻¹, which equates to 0.15 cm ha⁻¹ year⁻¹. Although this may seem minimal, the top layers of soil are where the majority of soil organic matter and biological activity occurs. Over half of the farm-induced soil erosion in the United States occurs in the Corn Belt.
and an area to the west, known as the Northern Plains (USDA, 2011). Both the Corn Belt and the Northern Plains are where crop production is most intense in the United States.

Conventional crop production utilizes large quantities of pesticides, herbicides, and fertilizers. Still relatively cheap, these products are an economical means to improving crop yields. However, the use of pesticides has a residual negative effect on watersheds. Traces of pesticides and their degrading byproducts are found in most streams and surface waters, thereby degrading water quality (Gilliom, 2006). Human exposure to pesticides may lead to various cancers and also cause reproductive, dermatological, neurological, and mental defects (Das et al., 2001; Eskenazi et al., 2007; Pearce and McLean, 2005). The synthesis and use of fertilizers is altering the global nitrogen cycle and contributing to the eutrophication of coastal waters (Nixon, 1995; Vitousek et al., 1997). Excessive use of pesticides and fertilizers degrades soil chemistry, decreasing overall soil quality (National Research Council, 2010). Soil quality also decreases when the soil structure is compacted by heavy farming machinery, leading to lower water and nutrient holding capacity and cycling. When soil quality suffers, this inevitably leads to lower production potential with higher production costs (Hardwood, 1994).

Conventional animal production makes use of antibiotics, medicated feeds, and growth hormones. Antibiotics and hormones from animal waste infiltrate the soil and contaminate surface and groundwater (Arikan et al., 2009; Kemper et al., 2008). This problem could be more widespread if the manure is applied as organic fertilizer to nearby or distant croplands (Kumar et al., 2005). Heuer et al. (2011) compile a review of antibiotic resistance in bacterial isolates from manure or manure-contaminated surface
water and states that this problem is spreading as application of fecal manure has become an alternative to commercial fertilizers in agricultural crop production. There is growing evidence that prophylactic use of antibiotics in animals is contributing to the spread of antibiotic resistant bacteria in humans (CDC, 2013). A recent publication by The Center for Disease Control (CDC) reports there are more antibiotics sold for food-producing animals in the United States than for human use (FDA, 2011). The CDC document further states that “resistant bacteria can be transmitted from food-producing animals to humans through the food supply” (CDC, 2013). Excessive agricultural use of herbicides and pesticides on plants also results in the development of genetic resistance. Numerous plants are displaying increasing resistance to pesticide use. The number of resistant plant biotypes has increased from about 50 in 1980 to 383 in 2012 (Adler, 2011; Heap, 1997).

1.3. Solutions

The National Research Council (2010, p.21) has compiled a list of strategies to improve the environmental impacts of conventional agriculture which include; crop rotation, cover crops, reduced and/or no-tillage, integrated pest management, precision farming (which aims to increase biodiversity), diversification of farm enterprises, genetically modified crops, and agricultural conservation best management practices. This dissertation offers an additional strategy to improve the environmental impacts of conventional animal production by implementing the use of PSM in grazing systems.

Plants naturally produce primary and secondary metabolites. Primary metabolites are affiliated with the growth and development of plants. They provide the necessities required for processes such as photosynthesis, respiration, and translocation. Plants also
produce metabolites that are not directly involved in growth and development, known as secondary metabolites. Based simply on the biosynthetic pathway of plant secondary metabolite production, there are three major classes; (i) phenolic compounds, (ii) terpenes and steroids, and (iii) nitrogen-containing compounds (Verpoorte, 1998). This research project covers the three major classes and focuses specifically on condensed tannins (phenolic compounds), saponins (terpenes), and the nitrogenous alkaloids gramine and ergovaline.

Plant secondary metabolites are well known for their toxic effects on animals. However, research over the past four decades has illuminated the ecological importance of these metabolites. Plant secondary metabolites provide numerous benefits to the plant itself by attracting pollinators and seed dispersers, helping plants recover from injury, protecting plants from ultraviolet radiation, and aiding in defense against pathogens, diseases, and herbivores. Secondary metabolites such as tannins may also contribute to drought tolerance by increasing elastic resilience in cell walls (Herms and Mattson, 1992; Taiz and Zeiger, 2002).

Until recently PSM were considered metabolic waste products rendering foods and forages poisonous and therefore unpalatable for consumption by humans and other animals. However, as Paracelsus (1493-1541) quotes, “All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy.” Some plants are more toxic than others, and some animal species are more susceptible to certain toxins than others. However, appropriate doses of certain secondary metabolites or ‘toxins’ offer health benefits to animals (Cheeke, 1998; Engel, 2002). For instance,
resveratrol, a polyphenolic compound in grapes, wine, soy and peanuts, helps prevent heart disease and various types of cancer in humans (Crozier et al., 2001).

Secondary metabolites also offer value to the health of ruminants. Saponins lower cholesterol in animals (i.e. Aazami et al., 2013) while phenolic compounds have antioxidative and anticarcinogenic benefits, and aid in digestion (Waghorn, 2008; see Waghorn et al., 1994). Condensed tannins reduce internal parasites and nematodes in ruminants and, due to their protein-binding characteristics, also enhance the absorption of amino acids in the small intestine (Barry and McNabb, 1999; Villalba et al., 2013). In addition, the consumption of tannins may reduce the emission of methane gas from ruminants (Jayanegara et al., 2009).

Plant secondary metabolites influence soil decomposition processes (Smolander et al., 2012). Tannins and terpenes are known to affect both carbon and nitrogen cycling in the soil, particularly by increasing nitrogen immobilization (Bradley et al., 2000). Nitrogen and carbon are immobilized and sequestered together, therefore incorporating tanniferous forages into ecosystems (e.g. pasture systems) may increase carbon sequestration potential. The global C stock in temperate grasslands, shrublands, and croplands are among the lowest in the world (Horwath, 2007). Increasing carbon sequestration potential in these ecosystems would be advantageous to our current carbon crisis. In the United States roughly 55% of the land is grazed, and this proportion is comparable on a global scale (Smith and Collins, 2007). Even without incorporating tanniferous forages, properly managed grazing systems have been shown to increase soil carbon as opposed to ungrazed systems (Clark et al., 1998; Smith and Collins, 2007).
2. Aim of the dissertation

This dissertation seeks to increase our understanding of PSM and implement their value in animal husbandry by determining; (i) how planting configuration (monocultures vs. two-way mixtures) influences PSM and total N in plants, as well as soil nutrient cycling, (ii) how cattle grazing PSM influence soil quality and nutrient cycling, and (iii) how cattle manure from different diets containing PSM influences soil characteristics. This dissertation also assesses whether forage crops produce sufficient PSM to influence soil nutrient cycling.

3. Literature review

3.1. Soil

Soil may be described as the foundation of life as no species, plant or animal, would exist without it. Throughout history, healthy soil reflects the health of the people living on and from it. Within the scientific community the terms soil health and soil quality have been used interchangeably. The definition of soil health has been explored and written about extensively, and is thought to be the ability for living soil to function and sustain plant and animal productivity and health as well as enhancing water and air quality (Doran, 1994; Doran and Parkin, 1996; Doran and Safley, 1997).

The ability for a soil to function optimally or provide desired ecological services depends largely on soil organic matter (SOM), which affects the physical, chemical, and biological properties of soil (i.e. Horwath, 2007). Soil organic matter consists of living material such as plants and soil macro and microorganisms, as well as any material that once lived, whether plant or animal. Deceased material exists in varying degrees of
decomposition. Slightly decomposed organic matter is usually at the surface and consists mostly of plant litter. Plant litter is further decomposed by soil macro and microorganisms into humus, the material that has been decomposed to the furthest extent. The source and quality of plant litter influences the quality and function of soil organic matter. Organic compounds decompose in the soil at different rates. Lignins and phenolic compounds decompose very slowly while sugars, starches, and simple proteins decompose quite rapidly (Brady and Weil, 2002). Slower decomposition can result in both accumulation of organic matter, and the inhibition of N mineralization and C oxidation in the soil.

Soil organic matter formation involves four major factors; climate, input and quality of organic material, existing SOM mineralization rates, and soil texture (Johnston et al., 2009). Climate has an important role in SOM formation, which tends to be higher in cooler regions, as cooler temperatures slow microbial activity. Soil organic matter also tends to be higher with increasing soil moisture. However, these generalizations have many other influencing factors. The focus of this dissertation lies mainly with the input and quality of organic material.

Plant tissues comprise the majority of what eventually builds SOM. On average, dry matter in plants contains sugars and starches (5%), simple and crude proteins (8%), hemicellulose (18%), cellulose (45%), fats and waxes (2%), lignins (20%), and polyphenols (2%) (Brady and Weil, 2002). However, there is substantial variance between plant species, different plant parts, and fluctuating growth stages. Specific chemical composition of a plant, and the amount of subsequent litter, influences soil
dynamics. Wedin and Tilman (1990) discovered that even among different grass species there were significant differences in soil N cycling.

Soil organic matter affects the physical and chemical properties of the soil. Physical indicators of soil health include the structure of soil. Soil organic matter binds soil particles into aggregates which allows adequate water infiltration rates, water holding capacity, and porosity which influences rooting depth of plants as well as creating ideal habitat for soil microbes (Horwath, 2007). Another key feature of soil organic matter is the existence of the smallest of soil particles; the clay and humus colloids.

Colloids (Greek = glue-like) are soil particles with diameters less than 1 micron in size, have a very large surface area per unit of mass and are electrically charged. This electric charge, whether positive or negative, allows both adhesion of cations and anions to colloid surfaces, and the exchange of ions in the soil solution. The capacity of soil to exchange cations (CEC) is a chemical indicator of soil health. Another important chemical feature of soil is the hydrogen ion concentration (acidity) of the soil solution – measured as soil pH. Many chemical and biological reactions depend on the acidity or alkalinity of the soil. Nutrient availability is strongly influenced by pH, which is optimal between 6.5 and 7.5, the range generally best for plant uptake and immobilization by most bacteria (Brady and Weil, 2002).

A healthy soil ecosystem is literally a living soil, containing a wealth of organisms such as bacteria, fungi, nematodes, protozoa, earthworms, and arthropods that function as a complex food web. These organisms are responsible for the life cycle in the soil, which supports the life cycle of plants through nutrient retention and recycling to decomposition. The balance of C and N in the soil is critical for the organisms living in
the soil. If organic matter consists of a mass-based carbon/nitrogen (C:N) ratio that is too high (e.g. 25:1), it may inhibit decomposition rates as well as decrease the amount of N available for plants (Brady and Weil, 2002). The decomposition of plant residue and organic material, microbial N-fixation, and nutrient availability is a series of processes involving predator-prey interactions of soil organisms (Kennedy and Papendick, 1995). Smaller creatures such as bacteria and fungi decompose plant residues, locking up nutrients from the decomposed material within their bodies. Bacteria and fungi are constantly being consumed by larger predators such as protozoa and nematodes, releasing these vital nutrients into the soil. The majority of dynamic relationships between soil organisms occur as a result of adequate enzyme activity.

Enzymes can be found in the soil solution, which is the aqueous phase of the soil where a variety of solutes and ions exist. Enzymes, also found in soil microbes or in the guts of small animals, carry out the majority of biological processes in the soil (Kandeler, 2007). In fact, an important biological indicator of soil health or soil quality is the activity of enzymes such as dehydrogenase and urease in the soil (Chellemi and Porter, 2001). Dehydrogenase is an enzyme that catalyzes oxidation and reduction reactions, while urease catalyzes the hydrolysis of urea to form ammonium carbonate. Phenol oxidase, another soil enzyme, oxidizes phenolic compounds and consumes oxygen (Sinsabaugh, 2010).

Research on the role of PSM in the realm of soil has increased over the past decade, particularly in silviculture systems, yet there are still numerous questions to be answered. Plant secondary metabolites affect decomposition rates, soil micro- flora and fauna, C and N cycling, and enzyme activity (Bradley et al., 2000; Lorenz et al., 2000;
Franzluebbers and Hill 2005; Joanisse et al., 2007; B. Adamczyk et al., 2009; S. Adamczyk et al., 2013; D’Addabbo et al., 2011; Smolander et al., 2012). Therefore, when attempting to manage agroecosystems it is important to consider the plant species and their intrinsic secondary metabolite chemistry and how these metabolites (and combinations of metabolites) might influence soil dynamics. Although research has investigated PSM in animal agricultural systems, there is virtually no research regarding PSM and soil dynamics in animal agriculture systems. This dissertation includes plant species that produce condensed tannins (phenolic compounds), saponins (terpenes), gramine and ergovaline (N-containing alkaloids).

3.2. Plant secondary metabolites in soil

Phenolic compounds, which include tannins, contain a hydroxyl functional group on an aromatic ring and can range in molecular weight from 500 to 3000 daltons. Condensed tannins are polymers of three-ring flavonols joined with C-C bonds (Salminen and Karonen, 2011; Taiz and Zeiger, 2002). As these compounds are quite large, phenolic root exudates have been known to inhibit nitrification in soil by inhibiting N fixing bacteria such as *Nitrosomonas europaea*, and the rate at which plants exude these inhibiting compounds depends on soil pH and plant growth stage (AlSaadawi, 1988; Subbarao et al., 2007; Zakir et al., 2008). Microorganisms may use some of the phenolic ring structures from lignin and polyphenols in the synthesis of stable soil organic matter (Brady and Weil, 2002).

Tannins themselves behave differently in soil depending on geography and plant source, their disappearance in soil differing within a six month period (Kelleher et al.,
2006; Lorenz and Preston, 2002). Perhaps the most studied PSM in soil are tannins, specifically in boreal forest systems. The tannin content of litter in forest systems is much higher than other ecosystems, is not easily decomposed, and heavily influences C and N mineralization (Smolander et al., 2012). Winder et al. (2013) found that N-fixing bacteria were greater with low- vs. high-tannin litter inputs. Research suggests that these carbonaceous compounds inhibit N mineralization thereby increasing N immobilization. However, whether N immobilization is carried out by the protein-binding power of tannins, microbial inhibition due to the complexity of tannin structure, or both, is still unknown. Although Kelleher et al. (2006) mentions research supporting that of condensed tannins being either adsorbed to soil minerals, bound to other proteinaceous substances, or transformed, the fate of condensed tannins in soil is still unclear. Salminen and Karonen (2011) explain the role pH has on tannin behavior in animals; specific tannins being more oxidatively reactive in higher pH, while other tannins being protein-precipitates in lower pH. Although some research has investigated the role soil pH has on tannins (Kraus et al., 2004; Northup et al., 1995) many questions remain.

Terpenes are made up of five-carbon units, whether two C-5 units (monoterpenes) or five C-5 units (triterpenes) (see Augustin et al., 2011). Saponins are triterpenes, containing 30 carbons. They are found in alfalfa and are structured with a steroidal backbone joined by glycoside side chains. These sugars are likely, and relatively rapidly, hydrolyzed by soil bacteria, leaving the steroidal backbone sapogenin medicagenic acid. Medicagenic acid glycosides can be inhibiting (depending on soil type) to seed germination of other species as well as fungus growth in the soil (Jurzysta and Oleszek, 1987). Smolander et al. (2012) reviewed the literature on monoterpenes in soil and
showed they behave similarly to tannins in soil; they both inhibit N mineralization and nitrification. The review also discusses the gaps in knowledge regarding the influence larger terpenes (triterpenes, tetraterpenes, and/or polyterpenoids) may have on soil dynamics. Smolander pursued this gap of knowledge to discover that indeed, higher terpenes (diterpenes) indicate similar patterns of decreased N mineralization and nitrification as seen with monoterpenes (S. Adamczyk et al., 2013). Interestingly, the study further discovered that diterpenes decreased fungal growth yet increased bacterial growth in soil. There are still numerous questions to pursue regarding the dynamics of terpenes, namely saponins, in the soil.

Nitrogen-containing PSM are well known for their toxicity to herbivores, as well as their pharmaceutical attributes (Taiz and Zeiger, 2002). Most of these N-containing compounds are synthesized from common amino acids, and some increase during herbivore attack (Baldwin, 1988; Karban and Baldwin, 1997). Endophyte-infected (E+) tall fescue, one of the most abundant forage crops in the United States, may contribute to more resistant soil organic matter, as alkaloids produced by the fungus (*Neotyphodium coenophialum*) may inhibit soil organisms and microbial activity (Franzluebbers and Hill, 2005). This may thereby increase soil organic C storage over time. Humphries et al. (2001) found that growth in earthworms (*Eisenia fetida*) was 3.6 times greater when fed E+ leaf litter than earthworms fed E- leaf litter.

3.3. Plant secondary metabolites in plants

The environmental milieu in which plants grow and interact influences their biochemical processes and constituents. There have been numerous hypotheses
attempting to generalize the influences on PSM concentration in plants (Berenbaum, 1995) such as; plant defense guilds (Atsatt and O’Dowd, 1976), plant apparency theory (Feeny, 1976), toxin/digestibility reduction continuum (Rhoades and Cates, 1976), optimal defense theory (McKey, 1974; Rhoades, 1979), carbon nutrient balance hypothesis (Bryant et al., 1983), resource availability hypothesis (Coley et al., 1985), environmental constraint hypothesis (Bryant et al., 1988), plant vigor hypothesis (Price, 1991), growth differentiation balance hypothesis (Herms and Mattson, 1992; Tuomi et al., 1984), and probability hypothesis (Jones and Firn, 1991). However, as useful as umbrella theories are there are always exceptions in nature.

Some studies have shown that N fertilizers can increase alkaloid levels in reed canarygrass, as well as other alkaloid-rich grasses (Majak et al., 1979). High alkaloid levels may also be a result of high temperatures, moisture stress, low light, as well as immature tissues (Majak et al., 1979; Marten, 1973; Thompson et al., 2001). While endophyte frequency and concentration may be higher in months with warmer temperatures and less precipitation, it is possible that the endophyte response is related to vernalization and physiological or morphological changes occurring in the plants (Ju et al., 2006). In southern Missouri ergovaline concentration in tall fescue was actually highest in mid-December and declined by 85% by the end of the winter (Kallenbach et al., 2003). Higher levels of CO₂ in the atmosphere can increase concentrations of carbonaceous compounds such as phenolics, while higher temperatures may reduce concentrations of phenolic compounds (Veteli et al., 2007). In some circumstances saponins in alfalfa, as well as condensed tannins in birdsfoot trefoil, fluctuate with the seasons; being high in the summer and low in the spring and fall (Cheeke, 1998). Yet in
another study total hydrolysable tannins in white birch tree leaves responded differently; being highest in spring, lowest in mid-summer, then increasing as the season ended (Salminen et al., 2001).

Fluctuations in PSM occur as a result of complex relationships involving production and allocation of resources within plants. One broad theory attempting to explain these fluctuations is the carbon/nutrient balance (CNB) hypothesis. This theory is based on the premise that nutrient deficiencies limit the rate of plant growth more than they limit the rate of photosynthesis (Bryant et al., 1983). When nutrients are curtailed, creating a high carbon-nutrient ratio, a plant may decrease its growth while still photosynthesizing at an undiminished rate, leading to an accumulation of carbohydrate in excess of what is needed to support immediate growth. This buildup of carbohydrate is believed to provide additional substrate for the production of carbon-based secondary metabolites such as tannins and phenolics. Conversely, under low-light conditions when growth is limited by the availability of C rather than nutrients, creating a low carbon-nutrient ratio, production of carbohydrates should decline, leading to a reduced formation of carbon-based defenses. The CNB hypothesis further suggests that abundant nutrient availability allows plants to accumulate excess N in addition to what is needed for primary growth. The N is then allocated to N-based secondary metabolites. Shade can also increase N-based secondary metabolites by decreasing growth (Herms and Mattson, 1992).

The CNB hypothesis can account for about 80% of the findings from experimental manipulations where plants have been fertilized or shaded (Reichardt et al., 1991), yet it does not account for all empirical observations (Hamilton et al., 2001;
Koricheva, 2002). The next iteration of this model, the growth differentiation balance hypothesis (GDB), was originally conceived in 1932 by plant biologist W. E. Loomis. Though the fundamental premise of GDB is similar to that of CNB in that there are physiological trade-offs between growth and differentiation processes including secondary metabolism, GDB is more broadly inclusive of differing intrinsic and extrinsic factors that affect resource allocation in plants (Herms and Mattson, 1992). From an evolutionary standpoint, both the CNB and GDB suggest that fast-growing plants differ chemically from slower-growing plants by allocating more energy on growth to out-compete surrounding plants. Slow-growing plants tend to have more chemical defenses and also are more suitable to survive adverse environmental conditions by making more efficient use of limited resources (Coley et al., 1985; Herms and Mattson, 1992).

Though clarifying in several circumstances, adhering to the generalization of such complexities in nature may be limiting to scientific advancement (Berenbaum, 1995; Koricheva, 2002). Haukioja et al. (1998) as well as Jones and Hartley (1999) illuminate key factors when assessing fluctuations of specific PSM; namely by considering the biosynthesis of specific secondary metabolites or their constituents. Jonathan Gershenzon, who has significantly expanded our understanding of plant secondary metabolite biosynthesis, indicates that the few essential components involved in the production of secondary metabolites are merely acetyl coenzyme-A, mevalonic and shikimic acid from primary plant metabolism, as well as proteins phenylalanine, tyrosine, tryptophan, lysine, and ornithine (Gershenzon, 1994).

Phenolic compounds are classified by containing at least one aromatic ring, with at least one hydroxyl functional group attached, and are further divided into either
flavonoids or non-flavonoids (Crozier et al., 2001). Flavan-3-ols are a subclass of flavonoids and are the most structurally complex group, which include monomers catechin, epicatechin, and the oligomeric and polymeric proanthocyanidins (condensed tannins) (Crozier et al., 2001). Phenolic compounds are produced either through the shikimate or malonate pathway (Taiz and Zeiger, 2002). Condensed tannins can be produced through either pathway, while other tannins such as ellagitannins are only produced through the shikimate pathway (Salminen and Karonen, 2011). Condensed tannins are believed to be the result of condensation polymerization of simple monomer units (Salminen and Karonen, 2011). The exact biosynthesis of these compounds is still not perfectly clear but the activity of two key enzymes, leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR), are consistent with the biosynthesis of proanthocyanidins in several species (Xie and Dixon, 2005).

The biosynthesis of terpenes is better understood than the biosynthesis of phenolic compounds. Terpenes are made up of 5-carbon isopentenyl diphosphate (IPP) compounds, and the amalgamation of these 5-carbon compounds and their isomer, dimethylallyl diphosphate (DPP), are what renders larger terpenes (Taiz and Zeiger, 2002). For instance, monoterpenes contain two 5-carbon compounds, whereas triterpenes contain six 5-carbon compounds. Terpene biosynthesis involves various enzymes deemed prenyltransferases which include isoprenyl pyrophosphate synthases, protein prenyltransferases, and prenyltransferases (Liang et al., 2002). Monoterpenes are formed by the union of IPP and DPP to form geranyl diphosphate (GPP); and GPP may then combine with another IPP to form farnesyl diphosphate (FPP) (Taiz and Zeiger, 2002). Triterpenes are formed by condensed polymerization of two FPP molecules to form
squalene (Crozier et al., 2001), which is catalyzed by squalene synthase (Blagg et al., 2002). Augustin et al. (2011) outline a schematic of the process from the combination of IPP and DPP to the formation of squalene, then saponins. Saponins are triterpene derivatives known as triterpene glycosides (Crozier et al., 2001). *Medicago sativa* (alfalfa) contains medicagenic acid, soyasapogenol A and B, and lucernic acid.

Nitrogen-containing compounds, namely alkaloids, have been studied extensively due to their toxic effects on humans and other animals, as well as their beneficial pharmacological attributes. The biosynthesis of N-containing compounds is a well-studied area. Numerous alkaloids are synthesized from a small pool of amino acids such as lysine, tyrosine, and tryptophan (Taiz and Zeiger, 2002). Alkaloid biosynthesis is complex, involving developmental regulation of genes and enzymes, as well as specific differentiation of cell types (Facchini, 2001). The focus of this dissertation involves an ergot alkaloid produced by the fungal endophyte *Neotyphodium coenophialum*, which lives symbiotically within the sheaths and seeds of tall fescue (Christensen et al., 2002) as well as the roots (Azevedo and Welty, 1995). Fleetwood et al. (2007) elucidate a complex cluster of genes required for the synthesis of ergot alkaloids, genes which encode the enzymes necessary for ergovaline production, including dimethylallyltryptophan (DMAT synthase) (Wang et al., 2004).

3.4. Landscape management

Conventional crop and animal production has caused soil, water, and animal health and quality to decline during the past century. One attempt to reduce the use of conventional fertilizers has been to increase use of fecal fertilizers. However, due to
residues detected in manure, the spread of hormones and antibiotic resistance could pose further environmental threats when manure from intensively raised animals is utilized in this way. Although economically efficient, conventional feedlot systems of rearing cattle are not environmentally sound. Conventional feedlot systems depend on large-scale corn production, as well as the use of antibiotics and anthelmintics. As previously mentioned, these products used for conventional feedlot systems are detrimental to the environment. Raising cattle on pasture is not as productive as a feedlot due to a lower rate of gain. However, research involving strategic uses of diverse forage species with differing secondary metabolites is slowly gaining ground. Herbivore health is greater, as is their intake of food, when offered various forages that differ in PSM concentrations (Burritt and Provenza, 2000; Freeland and Janzen, 1974; Lyman et al., 2012; Smith, 1959).

Reducing the use of antibiotics and medicated feed supplements is also imperative for environmental improvements from conventional animal production. Ongoing research shows the learned behavior sheep adopt in order to self-medicate for internal parasites by grazing tanniferous forages (i.e. Villalba et al., 2013).

Research and opinions on how pasture grazing impacts the environment are diverse. Cattle or sheep grazing can adversely or positively impact landscapes depending on the management. Grazing affects ecosystem structure and function, both above and below ground, (Trlica and Rittenhouse, 1993) and a grazed pasture is an interrelated ecosystem of soil, water, nutrients, plants, and herbivores. The plants an animal consumes influence the nutrient content of animal urine and feces, which in turn influences soil, water, nutrients, and plants. Animals grazing on a pasture add manure, which recycles nutrients back into the soil (McBee, 1971), increasing soil microbial C
and N (Wang et al., 2006). However, grazing may also decrease soil C and N pools (Golluscio et al., 2009). Grazing may cause soil compaction, and urine deposits may add to the eutrophication of water systems but both are “context-dependent” depending largely on stock density, soil type, and moisture (Fernández et al., 2010; Orwin et al., 2010). The influence grazing has on the environment depends on the environment in which grazing occurs, as well as management strategies.

When plants grow in mixtures, the diversity of PSM tends to complement each other, reducing dependence on synthetic fertilizers and pesticides (Provenza et al., 2007). The complementarities lie in the concept that animal intake of a particular forage is limited by the inherent PSM, balancing the impact of herbivory among plant species (Freeland and Janzen, 1974; Foley et al., 1999). Incorporating N-fixing legumes into grass pasture systems balances N and C mineralization and immobilization in the soil (Hooper, 1997; Hooper and Vitousek, 1998; Wardle et al., 1999; Zak et al., 2003), typically increases yield and quality of the two forages, and reduces the need for N input to the grass (Beuselinck et al., 1992; Danso et al., 1991; Mallarino et al., 1990; Zarrough et al., 1983). An increased level of secondary metabolites in plants reduces the need for pesticides, as their biological activities work against invasion of insects and/or pathogens (Taiz and Zeiger, 2002). Saponins (Adel et al., 2000; De Geyter et al., 2012) alkaloids (Luijendijk et al., 1996; Wink et al., 1995), and tannins (Barbehenn et al., 2009, 2006a, 2006b) are toxic to some insect species and therefore act as natural insecticides.

Input and quality of organic material, and subsequent SOM, depend much on the management implemented on a landscape. Conventionally managed systems, which often include intense tillage and few organic inputs, result in SOM declines (Johnston et al.,
Drinkwater et al. (1998) found that when comparing systems of management, conventional agriculture yielded more and subsequently returned more plant biomass to the soil than two other leguminous organic systems, yet the conventional system leached more N while the two organic systems retained more soil C.

We know very little about how different management strategies (e.g. various grazing practices, agricultural methods, and plant mixtures) may affect concentrations of PSM. Even less is known about how mixtures of pasture forages and their secondary metabolites might influence soil nutrient cycling.

4. Objectives and approach

Plant secondary metabolites help sustain plants, improve animal nutrition at appropriate doses, and may also influence soil dynamics in agroecosystems. Thus, it is important to expand our knowledge on what affects the concentration of these metabolites in plants, as well as what effect these metabolites may have on soil function. Whether concentrations are too high or low could inhibit foraging animals, both above ground herbivores and macro and microorganisms in the soil. This dissertation aims to better understand what influences the concentrations of these metabolites in different plants, and how these metabolites interact in the realm of soil. The objectives of this dissertation are to determine (i) how planting configuration (monocultures vs. two-way mixtures) affects concentration of PSM and total N in plants, (ii) how cattle grazing forages with PSM affects soil quality and nutrient cycling, and (iii) how cattle manure from different diets, containing different PSM, influences soil characteristics.
4.1. Plant species

Tall fescue (*Festuca arundinacea*) is a primary forage species in pastures throughout the U.S. and produces two types of alkaloids; those associated with the plant and those due to the fungus *Neotyphodium coenophialum*, which lives symbiotically in the intercellular spaces of sheaths, stems, leaves, and seeds of tall fescue (Rottinghaus et al., 1991). The importance of tall fescue is evidenced by its widespread abundance in agricultural use. This bunchgrass is among the hardiest of cool-season grasses as it can withstand a diverse onslaught of environmental hardships. In fact, various NRCS State offices recommend tall fescue for a variety of uses such as: conservation crop cover, herbaceous wind barrier, reclamation crop, prescribed grazing, pasture and/or hay, field borders, and/or filter strips. The ergot (fungal-derived) alkaloids in tall fescue have various detrimental effects on animals but compared to ‘uninfected’ tall fescue, endophyte-infected fescue has greater drought tolerance, pest resistance, tiller numbers, biomass, seed mass, seed numbers, and germination rates (Hill et al., 1991).

Tall fescue synthesizes two diazaphenanthrene alkaloids on its own, perloline and perlolidine (Powers and Ponticello, 1968) while endophyte-infected tall fescue may include the following alkaloids: loline, N-acetyl loline, N-formyl loline, lysergic acid, ergonovinine, ergovaline, ergonovine, ergosine, ergotamine, ergocornine, ergocryptine, and ergocristine (Cheeke, 1998; Crozier et al., 2001; Porter, 1995). Perloline and perlolidine are not thought to be major contributors to the infamous effects of fescue toxicosis. The two classes of metabolites that are responsible for fescue toxicosis are loline and ergopeptine, which are only found in endophyte infected tall fescue so therefore are likely derivatives of the fungus. The ergopeptine alkaloids have
vasoconstrictive and dopamine agonist properties so are therefore the main culprits of fescue toxicosis.

Fescue toxicosis causes a number of physiological effects across species but cattle and sheep appear to be the most severely affected. Physiological impacts include increased salivation, dull fur, elevated body temperature (hyperthermia), increased heart rates, fat necrosis, altered lipid metabolism, copper deficiency, changes in red blood cell volume and hemoglobin, immunological dysfunction, reduced pregnancy rates, lower milk production, and fescue foot (Stidham et al., 1982; Thompson et al., 2001). Fescue foot is a notorious clinical sign of fescue toxicosis caused by vasoconstriction, which limits blood supply to the extremities, usually associated with hypothermia. Fescue foot is a gangrenous condition in which the hooves or feet slough off in extreme cases, also affecting the ears and tails. Pathogenesis of fescue toxicosis begins from a damaged vascular endothelium. Thus, cattle grazing endophyte-infected tall fescue have lower intake and therefore lower weight gain. The reduced intake is exacerbated with increased environmental temperatures and during daylight hours (Stuedemann et al., 1985).

Unfortunately, there is no proven effective treatment for fescue toxicosis. In order to reduce the negative impacts of tall fescue yet maintain vigorous hardy forage, this dissertation proposes to incorporate alternative forages. More specifically, the addition of tanniferous legumes such as birdsfoot trefoil or sainfoin as they may help reduce the impact of fescue toxicosis, as tannins may bind to proteinaceous compounds like alkaloids (Catanese et al., 2014; Charlton et al., 2000; Okuda et al., 1982).

Although partial focus of this project is to address the problems associated with tall fescue, I also include reed canarygrass (*Phalaris arundinacea*), which also contains
alkaloids that may be toxic to livestock (Marten et al., 1976). Reed canarygrass is a cool-season grass with application in irrigated pastures due to its hardiness and high yield (Marten et al., 1979). In its wild form, it contains eight alkaloids—four derivatives of tryptamine, gramine, hordine, and two derivatives of β-carboline (Culvenor et al., 1964; Wilkinson, 1958).

Legumes such as alfalfa (*Medicago sativa*), sainfoin (*Onobrychis viciifolia*), and birdsfoot trefoil (*Lotus corniculatus*) also have application in irrigated pastures due to N fixing capabilities and complementary root profiles. Alfalfa contains glycosides such as saponins (Lu and Jorgensen, 1987), while sainfoin and birdsfoot trefoil contain tannins (Czochanska et al., 1980; Ramírez-Restrepo et al., 2005).

### 4.2. Hypotheses and expected results

#### 4.2.1. Grasses vs. Legumes - Mixtures or Monocultures

Fibrous root grass systems are denser and shallower than taproot leguminous systems, and contain greater root biomass in the uppermost layers of soil where most soil microbes reside. Greater plant root biomass results in more substrate for soil microbes to decompose. Grass systems tend to be N-limited which inhibits microbial activity and immobilizes N, reducing plant N-availability. This slows decomposition, which increases SOM formation over time. However, without available N plant growth declines, decreasing C input from plant biomass. Sustainable agricultural grass systems need a source of N.
Leguminous systems have less root biomass in the uppermost layers of soil and therefore do not provide as much substrate for soil microbes as grass systems. Yet leguminous systems fix N, decreasing the C:N ratio of the residues entering the soil, which results in higher N mineralization rates. Available N may be so abundant not all of it is utilized by the plants and microbes and hence may leach from the system. Thus it could be argued that from the microbial standpoint, leguminous systems are C limited.

I hypothesize that (i) grass-legume mixtures will be optimal for plant growth of both species, as legumes provide grasses increased access to N, and grass systems provide more food for soil microbial communities in leguminous systems, (ii) PSM concentrations will be greater in grass-legume mixtures, as more nutrients will be available for plant growth and development, (iii) N mineralization and immobilization will be more balanced (i.e. cycle sufficient N for plant growth and microbial activity, reducing excess loss) in grass-legume mixtures, and (iv) SOM formation will increase in mixtures, as plant growth and microbial activity in grass systems will benefit from the increased access to N from legumes and legume soil systems will benefit from the fibrous root system of grasses.

4.2.2. Plant Secondary Metabolites Influencing SOM Formation

Both tannins and terpenes are known to inhibit soil microbial activity in silviculture systems. In forest systems this is generally viewed as a negative aspect between plant detritus and soil communities, as decomposition in forests is inherently slow. However, in animal agricultural systems mineralization is quite rapid and nutrients are often lost from the system. Slowing decomposition processes in animal
agroecosystems would be beneficial, as this would increase SOM and reduce nutrient loss to the environment.

I hypothesize that tanniferous forages will inhibit soil microbial processes more than forages containing terpenes, as tannins bind proteinaceous substances. I also suspect that feces from cattle grazing forages containing tannins or terpenes will have traces of PSM, which will slow mineralization from feces and reduce N loss.

4.2.3. Different Land Management

This dissertation implements two different methods of fertilization which include (i) fecal manure by spreading or grazing, where plant biomass is removed from the system, or redeposited via feces in grazed plots, and (ii) commercial fertilizer, where plant biomass is hayed. Control plots have no inputs and plant biomass is hayed.

As previously mentioned, grasses have thick fibrous root systems which offer abundant substrate for soil microbes although competition for N is intense without additional N inputs. Leguminous systems contain less substrate for microbes yet there is more available N. According to Johnston et al. (2009), C:N ratios less than 40:1 release N as they decompose, and fecal manures typically fall below this ratio.

I hypothesize that (i) adding commercial fertilizer or fecal manures to grass or legume systems will boost microbial communities, as these additions increase resources available to soil microbes, (ii) plant biomass in commercially fertilized plots will be greater, and (iii) fecal manure will increase SOM formation over time.
I hypothesize that (i) PSM production will be greater in commercially fertilized plots, (ii) tannins and terpenes in each system will slow decomposition processes, yet (iii) tannins will inhibit soil processes more than terpenes.

I hypothesize that control treatments will be the least sustainable, with decreasing plant growth over time, as soil nutrients are continually depleted with no inputs to resupply microbial food sources. As a result, I hypothesize that PSM production will be lower in this treatment.

5. Materials and methods

5.1. Soil analyses

Soil samples were analyzed (Gavlak et al., 2003) for nitrate-nitrogen (NO$_3^-$ -N), using the Cadmium Reduction method (Method S-3.10), and ammonium (NH$_4^+$ -N; Method S-3.50), soil pH (Method S-2.20), electrical conductivity (EC; Method S-2.30), Olsen extractable P and K (Method S-4.10), DTPA-extractable Fe, Zn, Cu, and Mn (Method S-6.10). Soil samples were analyzed for dehydrogenase enzyme activity using methods described by Tabatabai (1994), and phenol oxidase enzyme activity as described by Prosser et al. (2011). Total and organic carbon was analyzed using a Skalar Primacs SLC model CS22 (Breda, Netherlands), using the two temperature (575 - 1035°C) method described by Chichester and Chaison (1992). Total N was analyzed using a Skalar Primacs Solid Sample TN Analyzer (Breda, Netherlands). Soil respiration was measured, with ratios between microbial biomass to organic carbon calculated to determine metabolic efficiencies (Anderson and Domsch, 1978, 1989; Davidson et al., 1987; Smith et al., 1985). Soil bulk density was measured, and soil porosity calculated,
using methods developed by Blake (1965) and described by USDA ARS NRCS Soil Quality Institute (2001).

5.2. Plant analyses

Plant samples were analyzed for total N using a Leco FP-528 nitrogen/protein Determinator (St. Joseph, MI). Total condensed tannins in birdsfoot trefoil and sainfoin samples were analyzed using methods described by Mantz et al. (2008) developed by Terrill et al. (1992) and Reed (1986). Reed canarygrass was analyzed for gramine as described by Clemensen et al. (2017) using methods developed by Anderton et al. (1999). Tall fescue samples were analyzed for ergovaline using HPLC methods developed by Rottinghaus et al. (1991) and Hill et al. (1993). Alfalfa samples were analyzed as described by Clemensen et al. (2017) using a modification of Lee et al. (2001) using methods developed by Patamalai et al. (1990) and Wall et al. (1952).

5.3. Field experiment establishment

Field experiments were conducted at Utah State University’s Agriculture Research Field Station in Lewiston, Utah at 41° 57' 4" N, 111° 52' 26" W. The study site consisted of; 1) Kidman fine sandy loam (KfA); a coarse-loamy, mixed, mesic Calcic Haploxeroll, and 2) Lewiston fine sandy loam (Ln); a coarse-loamy, mixed, mesic Aeric Calciaquoll.

In fall 2005 plots were seeded with endophyte-infected tall fescue (*Festuca arundinacea*) variety KY31 at 28 kg/ha, reed canarygrass (*Phalaris arundinacea*) (variety not specified) at 11 kg/ha, inoculated birdsfoot trefoil (*Lotus corniculatus*) variety Goldie at 6 kg/ha, and inoculated alfalfa (*Medicago sativa*) variety Vernal at 11
kg/ha. Field experiments 1 and 2 were conducted in 2007-2008. Plots were reseeded in spring 2009 with endophyte-infected tall fescue (*Festuca arundinacea*) variety KY31 at 28 kg/ha, inoculated alfalfa (*Medicago sativa*) variety Vernal at 11 kg/ha, and inoculated sainfoin (*Onobrychis viciifolia*) variety Shoshone at 33 kg/ha. Experiment 3 was conducted in 2010-2012.

5.4. Experiment 1 – mixtures vs. monocultures

The mixture vs. monoculture trial employed three separate studies; 1- tall fescue in monoculture, and in two-way mixtures with birdsfoot trefoil and alfalfa, 2- alfalfa in monoculture, and in two-way mixtures with tall fescue and birdsfoot trefoil, and 3- birdsfoot trefoil in monoculture, and in two-way mixtures with alfalfa or tall fescue. Each study was constructed as a randomized complete block design with a one-way treatment structure (mixture vs. monoculture) with time (am vs. pm) and month (July and August, 2008) as repeated measures.

The mixture vs. monoculture studies in 2008 consisted of collecting composite plant samples twice (July and August) in each block (n=3), in the morning and evening for each study. All plant samples were then freeze-dried, ground to pass a 1-mm screen with a Wiley mill grinder (Thomas Scientific, Swedesboro, NJ), then stored in sealed plastic bags at -20°C until chemically analyzed as described above.

5.5. Experiment 2 – different fertilizer treatments

The fertilizer trial consisted of four separate studies; 1- birdsfoot trefoil with fecal manure, conventional fertilizer, and control, 2- reed canarygrass with fecal manure, conventional fertilizer, and control, 3- tall fescue with fecal manure, conventional
fertilizer, and control, and 4-alfalfa with fecal manure, conventional fertilizer, and control. Each study employed a randomized complete block design with a one-way treatment structure (fertilizer) with repeated measures (month) over two years. For each study, both years were analyzed separately.

Composite plant samples were collected three times (August, September, and October) in 2007 for each study in each block (n=4). In 2008 composite plant samples were collected for each study in each block (n=4) four times (May, June, July, and September). Composite plant samples were processed and chemically analyzed as described above.

5.6. Experiment 3 – soil and plant responses in grazed systems

The grazing trial employed a split-plot design with whole plots in blocks (RCBD) with two factors. The whole plot factor was the legume planting of saponin-containing alfalfa with tall fescue or tannin-containing sainfoin with tall fescue, where the subplot factor was a planting of tall fescue grass, a legume, and a two-way mixture of grass/legume. Analyses for soil included 5 depths (0-10cm, 10-20cm, 20-30cm, 30-60cm, and 60-90cm); depth and its interactions with treatment were assessed separately by depth. Soil data for each year (2009, 2011, and 2012) was analyzed separately. The plant data includes repeated measures with three months of data collections (June, July, and August) over two years (2010 and 2012).

Before planting in summer 2009, composite baseline soil samples were collected in each block (n=3) using a Giddings probe to a depth of 90 cm and split into three increments (0-30, 30-60, and 60-90 cm). In spring 2011, composite soil samples were
collected in each block to a depth of 30 cm and split into three increments (0-10, 10-20, and 20-30 cm). In September 2012 composite soil samples were collected in each block using a Giddings probe to a depth of 90 cm, and split into five increments (0-10 cm, 10-20, 20-30, 30-60, and 60-90 cm). Soil samples were sieved to pass a 2-mm screen and stored at 4°C until analysis within 2 weeks. Soil samples were analyzed as described above.

In May 2010 composite baseline plant samples were collected in each grazed plot treatment. After forages were grazed in 2010 mixtures became predominantly tall fescue, as the grass outgrew each legume, confounding the analysis. Grazing proceeded through September 2010 while plant collections ceased, with attempts to reestablish mixture treatments by spreading more inoculated seed of both legume species in their respective plots. However, both legumes in mixture treatments failed to reestablish, therefore tall fescue and legume samples for mixture treatments were collected along the boundary of tall fescue and either alfalfa or sainfoin strips, where tall fescue and either legume were growing directly adjacent to each other. Due to over-winter crop failure (2010-2011 winter) alfalfa and sainfoin monoculture plots over all 3 blocks required reseeding in spring 2011, therefore the project resumed in 2012. From June – September 2011 treatments were swathed and baled. Composite plant samples were collected in each treatment three times in 2012. Sainfoin samples were placed on dry ice in the field, then stored at -20°C until freeze-dried. Alfalfa and tall fescue samples were placed in drying ovens at 30°C. Plant samples were analyzed as described above.
5.7. Experiment 4 - laboratory incubation study

To better understand how different cattle diets and the ensuing feces affects soil characteristics, the field research from experiment 3 was coupled with a laboratory soil incubation study. The goal was to determine if soil characteristics differ when grazing cattle are fed alkaloid-containing endophyte-infected tall fescue with either tannin-containing sainfoin or saponin-containing alfalfa.

In fall 2011, soil was collected and air-dried from the top 30 cm from the field experiment site. In July 2012 cattle feces was collected from experiment 3 where cattle were introduced to a diet of either tall fescue/sainfoin or tall fescue/alfalfa in early June, 2012. Composite samples of fresh feces were collected from each diet across three replications of grazed plots. A subset was analyzed for pH as described above, and total N and C using Leco FP-528 total combustion (St. Joseph, MI). Fresh fecal samples were then freeze dried, ground, and sieved through a 2 mm screen to be used in the soil incubation experiment.

The incubation study employed a block design with two factors with repeated measures, where feces from either alfalfa or sainfoin-based diets were mixed with soil at two different rates and incubated at 24°C for 56 days. Each treatment had four subsamples containing 500 g air-dried soil. Feces rates were based on (i) the assumption of daily excretions (Nennich et al., 2005), our low-rate treatment, and (ii) N fertilizer recommendations from N content of feces (Honeycutt et al., 2005), our high-rate treatment. Assumed excretions for the Angus fall-born calves were 3.74 kg DM day⁻¹, and calculations determining the amount of feces to be added to each subsample of soil (Honeycutt et al., 2005) used a bulk density of 1.3g cm⁻³.¹
On day 0, distilled water was added to each subsample of soil and feces to 18% moisture by slowly misting while mixing, then each subsample was slightly compacted to reach a bulk density of roughly 1.3g cm\(^{-3}\). The initial weight of all samples was recorded and moisture content monitored, adding distilled water when evaporation loss was > 5% (relative) of 18% moisture. Samples were placed in sealed quart-size Ziploc® bags with protruding straws to allow exchange of gases and minimize water loss, and stored at 24°C.

Sampling for analyses occurred at days 0, 3, 7, 14, 21, 28, 42, and 56. Samples were analyzed for NO\(_3\), NH\(_4\), dehydrogenase, organic C, and total N as described above. Total N and organic C was measured at the beginning and end of the incubation period (days 0 and 56). Two feces types, both at low- and high-rates, including the control, totaled 160 samples for analysis (5 treatments x 4 blocks x 8 sample days).

6. Impacts and outcomes

It is well known how beneficial PSM are to plants, and various studies have proven the benefits of moderate doses of these metabolites in animal diets (Crozier et al., 2001; Engel, 2002; Herms and Mattson, 1992; Taiz and Zeiger, 2002). Research also shows the benefits of these metabolites in the milk and meat of animals foraging on diverse plant species (Vasta et al., 2008; Vasta and Luciano, 2011). This dissertation will help expand our knowledge of how to manage pastures in order to utilize these natural metabolites, and how to better balance C and N cycling in pasture soil systems. Utilizing these natural metabolites may also help reduce reliance on fertilizers and pesticides in
pasture systems, as well as reducing reliance on antibiotics and anthelmintics for livestock.

My goal is to aid in the collaborative effort that demonstrates how strategic management may restore our latent fertile soil, improving the health of plants, herbivores, and people. In addition to publications for the scientific community, my research results and recommendations will be available to the public through the BEHAVE network (extension.usu.edu/behave).

Behavioral Education for Human, Animal, Vegetation, and Ecosystem management (BEHAVE) has been conducting scientific research and collaborating with land managers for the past 30 years. This group laid the foundations for what is now known as behavior-based management of landscapes. We are committed to bridging behavioral principles and processes with local knowledge to enhance ecological, economic, and social values of rural and urban communities and landscapes. We engage small and mid-sized producers, land managers, extension, and technical assistance personnel in a variety of education and outreach activities -- fact sheets, booklets, videos, slide shows, displays and demonstrations, seminars, workshops, short courses, formal courses, and symposia.

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CHAPTER 2
HOW PLANTING CONFIGURATION INFLUENCES PLANT SECONDARY METABOLITES AND TOTAL N IN TALL FESCUE (FESTUCA ARUNDINACEUS SCHREB.), ALFALFA (MEDICAGO SATIVA L.), AND BIRDSFOOT TREFOIL (LOTUS CORNICULATUS L.):
IMPLICATIONS FOR GRAZING MANAGEMENT

Abstract

Theories suggest that incorporating alfalfa (Medicago sativa L.; Alf) or birdsfoot trefoil (Lotus corniculatus L.; BFT) into endophyte-infected tall fescue (Festuca arundinacea Schreb.; E+TF) pasturelands may improve livestock production. We investigated how planting configuration might influence plant secondary metabolites (PSM) and nitrogen concentration in these forages. Total nitrogen (N), in addition to condensed tannins (CT), saponins, and ergovaline (EV) were compared in BFT, Alf, and E+TF, respectively, when forages grew in monocultures and all possible two-way “mixtures” using a block design with repeated measures in three blocks. Ergovaline ($P < 0.01$) and N ($P < 0.001$) concentration in E+TF was greater when growing adjacent to legumes than when growing in monoculture, and N and EV concentrations in E+TF were positively correlated ($r = 0.51; P = 0.001$). No differences in saponins or CT were found when Alf or BFT grew in monoculture or in two-way mixtures ($P > 0.10$). We conducted an in vitro trial to determine if CT or saponins isolated from BFT and Alf, respectively, would bind to EV in water. Ergovaline bound with saponins to a greater extent (39%; SE = 0.25) than CT (5.1%; SE = 5.13; $P < 0.05$). Because EV was lower in E+TF
monocultures than in E+TF-legume mixtures, and because CT and saponins may form complexes with EV which may alleviate fescue toxicosis, animals may benefit from systems which allow them to graze monoculture patches of E+TF and Alf or BFT rather than grass-legume mixtures.

1 | INTRODUCTION

Endophyte-infected tall fescue (E+TF) is a primary forage species in pasturelands throughout the U.S. and is among the hardiest of cool-season grasses as it withstands diverse environmental hardships. Endophyte-infected tall fescue has greater drought tolerance, pest resistance, tiller numbers, biomass, seed mass, seed numbers, and germination rates than uninfected tall fescue (Hill, Belesky, & Stringer, 1991). Although the fungal endophyte, *Neotyphodium coenophialum*, confers several agronomic advantages to tall fescue, it also produces toxic ergopeptide alkaloids which cause fescue toxicosis in livestock grazing E+TF. Fescue toxicosis causes a number of physiological effects across species but cattle and sheep appear to be the most severely affected (Stidham, Brown, Daniels, Piper, & Fetherstone, 1982; Thompson, Stuedemann, & Hill, 2001). Cattle grazing E+TF have lower intake and therefore lower weight gain. The reduced intake is exacerbated by higher environmental temperatures and during daylight hours (Stuedemann et al., 1985).

Fescue toxicosis is a widespread problem. The two classes of alkaloids responsible for fescue toxicosis are loline and ergopeptide, which are metabolites produced by the fungus. The ergopeptide alkaloids (particularly ergovaline; EV) have vasoconstrictive and dopamine agonist properties (Wink, 2010). Experiments using
dopamine antagonist therapy to mitigate the effects of fescue toxicosis have had some success (Aldrich, Rhodes, Miner, Kerley, & Paterson, 1993; Paterson, Forcherio, Larson, Samford, & Kerley, 1995; Rhodes, Paterson, Kerley, Garner, & Laughlin, 1991). However, dopamine antagonists merely prevent animals from experiencing the adverse effects of alkaloids, presumably experienced as nausea, thus ‘fooling’ animals into eating more fescue than their bodies can tolerate (Provenza, Ortega-Reyes, Scott, Lynch, & Burritt, 1994). In vivo research has found that grazing time by sheep and cattle on E+TF increases by incorporating saponin-containing alfalfa (Alf) or tannin-containing birdsfoot trefoil (BFT) into their diets (Jensen, Provenza, & Villalba, 2014; Lyman, Provenza, & Villalba, 2008; Lyman, Provenza, Villalba, & Wiedmeier, 2011; Owens, Provenza, Wiedmeier, & Villalba, 2012a,b). Increased grazing time on E+TF in the aforementioned studies could be due to a dilution effect as increased consumption of other forages would reduce intake of E+TF toxins. However, previous research suggests polyphenols (tannins) and saponins likely bind to N-containing molecules like alkaloids (Catanese, Distel, & Villalba, 2014; Charlton et al., 2000; Freeland, Calcott, & Anderson, 1985; Okuda, Mori, & Shiota, 1982), which could mitigate fescue toxicosis. Alfalfa contains the saponins medicagenic acid, soyasapogenol A and B, and lucernic acid, whereas birdsfoot trefoil contains condensed tannins (CT), also known as proanthocyanidins (Cheeke, 1998).

The effects of planting configuration on plant secondary metabolites (PSM) is less clear. Tall fescue growing adjacent to legumes may lead to greater concentrations of alkaloids as bacteria on the root nodules of legumes fix N. This natural source of available N from legumes increases the nutritional value of nearby grasses and also may provide a substrate to increase alkaloids in tall fescue (Arechavaleta, Bacon, Plattner,
Previous studies have found that tannin concentrations are greater when legumes grow in mixtures with tall fescue than in monocultures (MacAdam, Griggs, Beuselinck, & Grabber, 2006; Wen et al., 2003). We investigated how planting configuration of BFT, Alf, and E+TF influenced concentrations of CT, saponins, and EV, respectively. We also determined the binding capacity of purified CT and purified saponins with EV using an *in vitro* test. We hypothesized that concentration of PSM would be lower in monocultures than in grass-legume “mixtures” due to lower availability of nutrients to plants, and that saponins and CT would bind to EV in vitro.

2 | MATERIALS AND METHODS

2.1 | Field experiments: How does planting configuration influence plant secondary metabolites?

2.1.1 | Plot establishment

Research plots were established in fall 2005 at Utah State University’s Agriculture Research Field Station in Lewiston, Utah (41° 57' 4" N, 111° 52' 26" W). The study site consisted of Lewiston fine sandy loam, a coarse-loamy, mixed, mesic Aeric Calciaquoll. Four plots were seeded as pure stands in adjacent north-south strips (12x180 m) from west to east with: 1) inoculated Alf variety ‘Vernal’ at 11 kg ha⁻¹, 2) inoculated BFT variety ‘Goldie’ at 6 kg ha⁻¹, 3) E+TF variety ‘KY31’ at 28 kg ha⁻¹, and 4) a duplicate strip of Alf to provide a two-way “mixture” of E+TF/Alf. Plots were split from north to south into three experimental blocks.
2.1.2 | Sample collection and analysis

From each plant species (Alf, BFT, and E+TF) multiple subsamples (n=10) were clipped at 5 cm above ground then composited, from each monoculture and two-way “mixture”. Samples from each forage were selected in a simple random approach, both in the morning (0800-1000 hours) and evening (1800-2000 hours) in July and August, 2008. Each monoculture (Alf, BFT, and E+TF) was sampled at the center of each strip, away from other forage species. Each two-way “mixture” (Alf / E+TF, Alf / BFT, BFT / E+TF) was sampled along the boundary of forage strips, where two forage species were growing directly adjacent to each other. Plant samples from each species from the two-way “mixture” area were collected in separate bags. After sampling periods, plots were swathed and baled, then regrowth was allowed between July and August collections. Plant samples were gathered when developmental morphology was similar at both collection times (July 10, and August 21) for each species (Flick & Mueller, 1989; Moore & Moser, 1995; Moore et al., 1991). Plant samples were placed on dry ice in the field, then stored at -20°C until freeze-dried. Dried plant samples were ground to pass a 1-mm screen with a Wiley mill grinder (Thomas Scientific, Swedesboro, NJ), then stored in sealed plastic bags at -20°C until chemical analyses. Each plant species included 36 composite samples for analysis (3 treatments x 2 months x 2 diurnal periods x 3 blocks).

Two subsamples were taken from each composite plant sample for independent analysis for total N using a Leco FP-528 nitrogen/protein Determinator (St. Joseph, MI), and a mean value was computed over the two subsamples. Birdsfoot trefoil samples were analyzed for CT as described by Mantz, Villalba, and Provenza (2008) using methods developed by Terrill, Rowan, Douglas, and Barry (1992) and Reed (1986). Tall fescue
samples were analyzed for EV using HPLC methods developed by Rottinghaus, Garner, Cornell, and Ellis (1991) and Hill, Rottinghaus, Agee, and Schultz (1993). Alfalfa samples were analyzed as described by Clemensen et al. (2017) using a modification of Lee, Stegelmeier, and Gardner (2001) using the methods developed by Patamalai, Hejtmancik, Bridges, Hill, and Camp (1990) and Wall, Eddy, McClennan, and Klumpp (1952).

2.1.3 | Statistical Analysis

Data were analyzed as a randomized complete block design with three replications, using a 1-way treatment structure (mixture 1, mixture 2, and monoculture) with time (morning and evening) and month (July and August, 2008) as repeated measures. The response variables were CT %, saponin %, EV (ppb), and N %. Analyses were conducted using SAS PROC GLIMMIX (SAS Institute, Cary, NC). Differences between LS means were adjusted for family-wise Type I error using the Tukey-Kramer method. To assess the linear association between EV and N in E+TF, the Pearson correlation coefficient was estimated on N and EV data from all 36 samples using SAS PROC CORR (SAS Institute, Cary, NC).

2.2 | Laboratory experiment: Assessing whether tannins or saponins bind ergovaline in vitro.

In Alf and BFT monocultures across all three blocks from the field experiment site, multiple subsamples (n=25) from each species were clipped at 5 cm above ground in a simple random approach when both species were at late vegetation maturity (Flick and Mueller, 1989), then composited. Samples were placed on dry ice in the field then stored...
at -20°C until freeze-dried. Dried plant samples were ground to pass a 1-mm screen with a Wiley mill grinder, then stored in sealed plastic bags at -20°C.

Condensed tannins were isolated and purified from BFT as described by Mantz et al. (2008) using methods developed by Terrill et al. (1992) and Reed (1986). Saponins were isolated and purified from Alf using a modification (Clemensen et al., 2017) of Lee et al. (2001) using methods developed by Patamalai et al. (1990) and Wall et al. (1952). Purified saponins and tannins were stored at -20°C.

A primary EV (Veterinary Medical Diagnostic Laboratory, Columbia, MO) stock solution (1000 ppm) was prepared in acetonitrile. Ergovaline solutions for in vitro adsorption experiments were prepared by adding acetonitrile stock solution to 0.1M phosphate buffer adjusted to pH 7. The concentration of EV used in this study was 400 ppb. Duplicate aliquots of 0.1M phosphate buffer (pH 7) containing 400ppb EV in solution (10 mL) were added to 15 mL polypropylene Falcon tubes to which 100 mg of each tannin or saponin was added. To eliminate exogenous peaks, controls were prepared by adding 10 mL of 0.1 M phosphate buffer (pH 7) to Falcon tubes containing 100 mg of each adsorbent. All samples were placed on a rotator shaker for 30 min at room temperature and centrifuged for 5 min at 13000 rpm. 2 mL of the aqueous supernatant was removed for HPLC analysis. HPLC analyses were performed on a Hitachi L-7100 (Hitachi Instruments, Inc., San Jose, CA) pump with a Hitachi L-7200 autosampler, and fluorescence detection with a Hitachi L-7480 fluorescence spectrophotometer (λ.ex= 250 nm and λ.em = 420 nm). The column was a 10 cm X 5 um Luna C_{18} column (Phenomenex, Torrance, CA); the mobile phase was acetonitrile:water (35:65) with 200
mg ammonium carbonate/L, pumped at the flow rate of 1 mL/min. Data were recorded and processed by a Hitachi D-7000 data acquisition package with Concert Chrom software on a microcomputer. An aliquot of the original buffered EV test solution was used as the HPLC standard. The percentage of EV bound was calculated by the difference of the initial and final concentration in the aqueous supernatant after equilibrium was reached.

2.2.1 | Statistical Analysis

Means of percent of bound EV, when combined in vitro with CT extracted from BFT or saponins extracted from Alf, were compared using a two-sample t-test. Analyses were conducted using SAS PROC TTEST (SAS Institute, Cary, NC).

3 | RESULTS

3.1 | Field experiments: How does planting configuration influence plant secondary metabolites and total N?

3.1.1 | E+ tall fescue grown in monoculture or adjacent to birdsfoot trefoil and alfalfa

Ergovaline concentration means in E+TF were greater (P = 0.01) when plants grew in “mixtures” with BFT (316 ppb) and Alf (299 ppb) than when E+TF grew as a monoculture (133 ppb; SE = 29.3), and none of the interactions were significant. There were no changes in EV concentration from morning to evening (P = 0.53), or from July to August (P = 0.32).

Total N means in E+TF were greater (P = 0.001) when the plant grew adjacent to BFT (2.58%) and Alf (2.36%) than when grown as a monoculture (1.84%; SE = 0.04),
and the interaction with month was significant (P = 0.001). Total N in E+TF tended (P = 0.052) to be greater in the morning (2.31 %) than the evening (2.20 %; SE = 0.03), but none of the interactions were significant. The month within treatment interaction was significant (P = 0.001), showing N increasing (P = 0.04) from July to August when E+TF grew adjacent to BFT, while decreasing (P = 0.003) from July to August when E+TF grew adjacent to Alf, with no differences in total N between July and August when E+TF grew in monoculture (Table 2-1). The correlation coefficient between EV and N concentration in E+TF was 0.51 (P = 0.001) indicating there was a significant positive relationship between N content and EV concentration in this study.

**TABLE 2-1** Month within treatment means and standard errors (SE) of total N % in endophyte-infected tall fescue (E+TF; P < 0.01), alfalfa (Alf; P < 0.01), and birdsfoot trefoil (BFT; P = 0.11) in monoculture and two-way mixtures (w/ ‘forage’) from July to August, 2008. * P < 0.05; ** P < 0.01.

<table>
<thead>
<tr>
<th>Forage - Treatment</th>
<th>July</th>
<th>August</th>
</tr>
</thead>
<tbody>
<tr>
<td>E+TF - monoculture</td>
<td>1.80 (0.06)</td>
<td>1.87</td>
</tr>
<tr>
<td>E+TF - w/ BFT</td>
<td>2.39 *</td>
<td>2.78</td>
</tr>
<tr>
<td>E+TF - w/ Alf</td>
<td>2.69 **</td>
<td>2.02</td>
</tr>
<tr>
<td>Alf - monoculture</td>
<td>4.53 (0.09)</td>
<td>4.74</td>
</tr>
<tr>
<td>Alf - w/ BFT</td>
<td>4.38 *</td>
<td>4.88</td>
</tr>
<tr>
<td>Alf - w/ E+TF</td>
<td>4.36</td>
<td>3.99</td>
</tr>
<tr>
<td>BFT - monoculture</td>
<td>3.71 (0.08)</td>
<td>3.99</td>
</tr>
<tr>
<td>BFT - w/ Alf</td>
<td>3.87</td>
<td>3.67</td>
</tr>
<tr>
<td>BFT - w/ E+TF</td>
<td>3.65</td>
<td>3.60</td>
</tr>
</tbody>
</table>

3.1.2 | **Birdsfoot trefoil grown in monoculture or adjacent to E+ tall fescue and alfalfa**

While means of CT in BFT appeared greater when the plant grew adjacent to E+TF (6.09%) and Alf (5.23%) than when grown in monoculture (4.45%), there were no significant differences among treatments (P = 0.25; SE = 0.52). Overall means of CT in
BFT were significantly (P < 0.001) greater in the morning (6.82%) than in the evening (3.69%; SE = 0.38). The time within month interaction was significant (P < 0.01), such that CT decreased from morning (5.88%) to evening (4.91%) in July, and showed a more distinct pattern in August (7.75% am; 2.47% pm; SE = 0.58).

Total N means in BFT did not differ (P = 0.10) when the plant grew adjacent to E+TF (3.63%), Alf (3.77%), or when grown in monoculture (3.85%; SE = 0.05). There were no differences in total N from July to August (P = 0.89). Overall means of N in BFT decreased from morning (3.84%) to evening (3.66%; P < 0.0001; SE = 0.02). The time within treatment interaction was significant (P = 0.003), such that diurnal differences in N means in BFT were more pronounced when BFT grew adjacent to Alf (3.94% am; 3.60% pm) and E+TF (3.71% am; 3.55% pm) than when BFT grew in monoculture (3.89% am; 3.82% pm; SE = 0.05).

3.1.3 | Alfalfa grown in monoculture, or adjacent to E+ tall fescue and birdsfoot trefoil

No differences in saponin concentration were found when Alf grew in monoculture or adjacent to E+TF or BFT (P = 0.26), nor were there diurnal differences (P = 0.79). There was a trend (P < 0.06) for saponin means to differ between months, appearing to decrease from July (3.27%) to August (2.37%; SE = 0.30).

Total N means in Alf were greater (P = 0.01) when the plant grew adjacent to BFT (4.63%) and in monoculture (4.63%) than when it grew adjacent to E+TF (4.18%; SE = 0.08), and the interaction with month was significant (P = 0.002). Total N means in Alf decreased (P < 0.0001) from morning (4.63%) to evening (4.33%; SE = 0.06). There was a trend (P = 0.09) for total N in Alf to be greater in August (4.54%) than in July.
(4.42%; SE = 0.06). The month within treatment interaction was significant (P = 0.002), showing that while N increased (P = 0.02) from July to August when Alf grew adjacent to BFT, N tended to decrease (P = 0.06) from July to August when Alf grew adjacent to E+TF, remaining unchanged from July to August when growing in a monoculture (P = 0.4; Table 2-1).

3.2 | Laboratory experiment: Assessing whether tannins or saponins bind ergovaline in vitro

Ergovaline bound with saponins (mean = 39.0%; SE = 0.35) isolated from Alf to a greater extent than EV bound with CT (mean = 5.13%; SE = 5.13) isolated from BFT (P = 0.02; t = 6.60; DF = 2; Table 2-2).

**TABLE 2-2** Percent of bound ergovaline (EV) when combined *in vitro* with condensed tannins extracted from birdsfoot trefoil and saponins extracted from alfalfa.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample</th>
<th>% Bound EV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condensed tannins</td>
<td>BFT (a)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>BFT (b)</td>
<td>10.25</td>
</tr>
<tr>
<td>Saponins</td>
<td>Alf (a)</td>
<td>38.75</td>
</tr>
<tr>
<td></td>
<td>Alf (b)</td>
<td>39.25</td>
</tr>
</tbody>
</table>

4 | DISCUSSION

We determined how planting configuration of E+TF, BFT, and Alf influenced PSM within the day and across time. We hypothesized that the concentration of each PSM would increase in grass-legume “mixtures”, assuming more nutrients would be available to plants. Consistent with this hypothesis EV concentration in E+TF was greater when the plant grew adjacent to Alf or BFT than when it grew in a monoculture. These
results are consistent with findings from a previous study where E+TF had greater EV concentrations when growing adjacent to Alf than when growing in monoculture (Friend, Provenza, & Villalba, 2015). Interestingly, Friend et al. (2015) also found that when sheep were given a choice between E+TF growing adjacent to Alf or in monoculture (with less EV), sheep preferred E+TF with greater EV which also had more crude protein, but only during the first hour of the feeding period. The correlation coefficient between EV and N in E+TF indicated a significant positive relationship which may imply that increased N renders greater EV in E+TF. However, correlation is not always causation and our studies did not delve into the physiological mechanisms behind this coefficient. Contrary to our hypothesis that PSM concentrations would be greater in “mixtures”, neither CT nor saponin concentrations differed significantly when plants grew in “mixtures” or in monocultures. The ability of saponins from Alf and CT from BFT to bind EV in vitro was evaluated in a water solution. We found that 39% of EV bound with saponins from Alf, which supports the theory that binding complexes may form between EV and saponins. Empirical grazing studies done by Lyman et al. (2011) found that grazing time on E+TF increased if cattle first grazed saponin-rich Alf, instead of the reverse sequence of grazing E+TF before Alf. Saponins isolated from Alf bound with EV to a greater extent than CT isolated from BFT. In contrast, recent in vitro studies suggest that CT in sainfoin (Onobrychis viciifolia) bind ergot alkaloids in E+TF (Villalba et al., 2016). Condensed tannins and saponins, each from various species, cause responses that vary substantially with the diversity of their chemical structures (Clausen, Provenza, Burritt, Reichardt, & Bryant, 1990; Francis, Kerem, Makkar, & Becker, 2002;
Collectively, the \textit{in vitro} results suggest that CT in sainfoin have greater affinity to bind to ergot alkaloids in E+TF than CT in BFT. Mixtures of N-fixing legumes and grasses typically increase yield and quality of the two forages while reducing recommended N inputs for the grass (Danso, Curbelo, Labandera, & Pastorini, 1991; Haynes, 1980; Mallarino, Wedin, Goyenola, Perdomo, & West, 1990), and can benefit landscapes (Sleugh, Moore, George, & Brummer, 2000; Suter et al., 2015). However, our results show that E+TF/legume “mixtures” increase EV concentration without significantly increasing CT in BFT or saponins in Alf. Developing a system where plants grow in strips as monoculture and animals have the option to graze different strips would be preferable as concentration of ergot alkaloids in E+TF monocultures would be reduced. Parsons, Newman, Penning, Harvey, and Orr (1994) found increases in forage intake and performance when sheep and cattle select forages in monoculture patches of different species as opposed to mixtures.

Planting separate paddocks of E+TF and legume monocultures while implementing sequence grazing may be best for animals grazing E+TF. Sequence grazing (i.e., rotational grazing) involves moving a herd through different paddocks containing different forages throughout the day. Various experiments have explored the benefits of sequence grazing across patches of different botanical composition and nutritional value (Lyman et al., 2011; Meuret, 1997; Meuret & Provenza, 2015). Extensive research at the French National Institute of Agronomic Research in Montpellier, France has examined the benefits of implementing sequence grazing over landscapes using a model deemed MENU (Meuret, 1997). The MENU model moves herds strategically through different paddocks, each of which act as a different meal phase (e.g., appetite stimulator, first and
second courses, dessert). A similar model could be applied for animals grazing E+TF and saponin-containing Alf.

Both CT and total N in BFT were greater in the morning than the evening. Total N content in Alf decreased from morning to evening, and saponin concentration in Alf tended to be greater (P < 0.06) in July than in August. Thus, time (diurnally and seasonally) is an important factor to consider when implementing grazing circuits. For instance, grazing legumes in the morning and Alf in July may enhance N and saponin intake, which would be beneficial for the nutrition of the animal and potential complexation of ergot alkaloids from E+TF, respectively.

Our field study results are limited by the short duration of the experiments. When studying ecological responses it is preferable to include multiple growing seasons. Wen et al. (2003) found greater CT in grazed BFT mixtures with tall fescue than in BFT monocultures over two growing seasons. However, their experiments used a different variety of tall fescue (cv. Phyter) which did not contain the endophyte *Neotyphodium coenophialum*. Our *in vitro* study is limited by using water instead of rumen fluid to evaluate binding capacity of EV with saponins and/or CT. Although there was significant binding of EV with saponins, we can only speculate that our results would apply *in vivo*.

In summary, we found no significant differences in CT or saponin levels in BFT or Alf (respectively), whether these legumes grew in monoculture or adjacent to E+TF. We observed that EV concentration increased when E+TF grew adjacent to both legumes. Our results show that timing, both short- and long-term, can affect total N levels and PSM concentrations in forages. We also discovered that EV binds with saponins from Alf *in vitro* using a water solution. From our studies we speculate that sequence
(rotational) grazing of monocultures of saponin-containing Alf followed by E+TF may assist in managing fescue toxicosis. Properly managed grazing circuits, which include a variety of plant species can be beneficial to grazing animals. Our experiments included two different legumes with theoretical intentions of alleviating fescue toxicosis. Further research investigating different forage combinations to reduce fescue toxicosis is needed, in addition to determining if binding of EV and saponins and/or CT actually occurs in the rumen.

REFERENCES


CHAPTER 3
PLANT SECONDARY METABOLITES IN ALFALFA, BIRDSFOOT TREFOIL,
REED CANARYGRASS, AND TALL FESCUE UNAFFECTED BY TWO
DIFFERENT N SOURCES

ABSTRACT
Plant secondary metabolites (PSM) may increase the sustainability of agriculture systems by reducing inputs, as PSM protect plants against herbivores and pathogens, act as pesticides, insecticides, and anthelmintics, while also attracting pollinators and seed dispersers. Therefore, it is important to understand what affects PSM fluctuation in plant tissues. Limited research has investigated how different nitrogen (N) sources affect PSM concentration in alfalfa (*Medicago sativa* L.; Alf), birdsfoot trefoil (*Lotus corniculatus* L.; BFT), reed canarygrass (*Phalaris arundinacea* L.; RCG), and endophyte-infected tall fescue (*Festuca arundinacea* Schreb.; E+TF). We investigated how fecal manure (feces) and synthetic N fertilizer (urea; 46% H₂NCONH₂) influence N concentrations and the PSM ergovaline, gramine, saponins, and extractable condensed tannins (CT) in E+TF, RCG, Alf, and BFT, respectively. Ergovaline, saponins, and CT were not affected by fertilization. Gramine tended (P = 0.06) to be greater in control plots than in fertilized plots. Total N in E+TF and RCG was greater (P < 0.05) and tended to be greater for Alf (P = 0.08) in synthetically fertilized plots than in unfertilized plots. Seasonal variation in PSM and N was significant (P < 0.003) across all species and it was species-specific. Total N in E+TF was greatest in June (41.4 g kg⁻¹) while ergovaline contents were at the lowest values recorded (117.2 μg kg⁻¹), with subsequent increases to the greatest
ergovaline values observed in July (680.0 µg kg\(^{-1}\)). Our results reveal the variability in PSM production by plants and highlight the complexities of predicting fluctuations of PSM in forages. As environments where plants grow vary through space and time, we recommend studies on a case-by-case basis depending on land management objectives.

**INTRODUCTION**

Plants produce primary and secondary metabolites. Primary metabolites are directly involved with plant growth and metabolism, whereas secondary metabolites protect plants against herbivores (arthropods, vertebrates, invertebrates), pathogens (viruses, bacteria, fungi), and plant competition via allelopathy. These compounds may also act as natural pesticides, insecticides and anthelmintics and they also attract pollinators and seed dispersers (Hartmann, 2007). Thus, PSM may increase the sustainability of agriculture systems by reducing inputs, therefore it is important to understand what affects their fluctuation in concentration within plant tissues.

Legumes such as alfalfa and birdsfoot trefoil are important perennial forage crops due to N-fixing capabilities. Alfalfa contains triterpene glycoside saponins (Lu and Jorgensen, 1987) which are derivatives of medicagenic acid, soyasapogenol A and B, hederagenin, and zanhic acid (Szakiel et al., 2011) whereas BFT contains condensed tannins, also known as proanthocyanidins (Cheeeke, 1998). Reed canarygrass is both a resilient and efficient cool-season grass (Marten et al., 1979) containing eight alkaloids; four derivatives of tryptamine, gramine, hordine, and two derivatives of β-carboline (Wilkinson, 1958; Culvenor et al., 1964). Endophyte-infected tall fescue is a primary forage species in pastures throughout the U.S. and is among the hardiest of cool-season
grasses. Endophyte-infected tall fescue produces two types of alkaloids; those associated with the plant (Powers and Ponticello, 1968) and those due to the fungus *Neotyphodium coenophialum* (Porter, 1994), which lives symbiotically in the intercellular spaces of sheaths, stems, leaves, and seeds of tall fescue (Rottinghaus et al., 1991; Thompson et al., 2001).

In addition to genetic, ontogenic and morphogenetic factors (Verma and Shukla, 2015), the environmental milieu in which plants grow and interact influences their biochemical processes and constituents. Environmental influences on plant secondary metabolite biosynthesis in plants varies across species, as well as within species (Verma and Shukla, 2015), complicating the umbrella theories attempting to explain these phenomena (Loomis, 1932; McKey, 1974; Atsatt and O’Dowd, 1976; Feeny, 1976; Rhoades and Cates, 1976; Rhodes, 1979; Bryant, et al., 1983; Tuomi et al. 1984; Coley et al., 1985; Bryant et al., 1988; Price, 1991; Jones and Firn, 1991; Herms and Mattson, 1992). Though illuminating in several circumstances, adhering to the generalization of such complexities in nature may be limiting to scientific advancement (Berenbaum, 1995; Koricheva, 2002). Despite such diversity of theories, limited research has been conducted on how different N sources affect PSM concentration in plant tissues. Studies have shown N fertilizers increase alkaloid levels in RCG (Majak et al., 1979), and E+TF (Rottinghaus et al., 1991; Arechavaleta et al., 1992).

Application of feces is an alternative to commercial fertilizers in agricultural crop production systems, and the practice is used throughout the world including the United States (Kumar et al., 2005). We investigated how feces and synthetic N fertilizer (urea) influence production of PSM in different forage species by measuring endophyte
produced ergovaline in E+TF, gramine in RCG, saponins in Alf, and CT in BFT. We hypothesized that 1) alkaloids ergovaline and gramine would initially be greater in urea fertilized plots as N is more readily available, and that alkaloids would increase more over time in feces fertilized plots as N is released more slowly, and 2) saponins and condensed tannins would be greater in plots fertilized with feces due to increased carbon availability to the plant.

MATERIALS AND METHODS

Plot Establishment

Research plots were established in fall 2005 at Utah State University’s Agriculture Research Field Station in Lewiston, UT at 41° 57’ 4” N, 111° 52’ 26” W. The study site consisted of; 1) Kidman fine sandy loam (KfA); a coarse-loamy, mixed, mesic Calcic Haploxeroll, and 2) Lewiston fine sandy loam (Ln); a coarse-loamy, mixed, mesic Aeric Calciaquoll. Three replicate blocks containing four forages each were selected from within a larger randomized study of 1) inoculated Alf variety ‘Vernal’ at 11 kg ha⁻¹, 2) E+TF variety ‘KY31’ at 28 kg ha⁻¹, 3) RCG ‘VNS’ (variety not specified) at 11 kg ha⁻¹, and 4) inoculated BFT variety ‘Goldie’ at 6 kg ha⁻¹. Subplots were chosen within each forage type per block, then three fertilizer treatments were applied at random to each forage type.

Soils were analyzed (Gavlak et al., 2003) for Olsen extractable P and K (Method S-4.10). Total and organic carbon was analyzed using a Skalar Primacs SLC model CS22 (Breda, Netherlands), using the two temperature (575 - 1035°C) method described by Chichester and Chaison (1992). Total N was analyzed using a Skalar Primacs Solid
Sample TN Analyzer (Breda, Netherlands). Soil tests revealed no differences in nutrients so fertilizer applications were based on N alone. Two E+TF replications were in Kfa while the remaining replications of the experiment were in Ln soil. Block effect was tested using SAS PROC SGPANEL (SAS Institute, Cary, NC) and we conclude that our measured responses in E+TF were not affected by the difference in soil type.

**Fertilizer Applications**

Plots were fertilized based on yield potential of 9 t ha\(^{-1}\) (Koenig et al., 1999; Koenig et al., 2002) on 16 August 2007, 21 May 2008, and 9 September 2008. Our study commenced late during the 2007 season therefore fertilizer was applied once. Plots were fertilized at a rate of 56 kg ha\(^{-1}\) N for E+TF and RCG plots, and 11.2 kg ha\(^{-1}\) N for Alf and BFT plots at each application time. Before each fertilizer application dry steer manure (1-3 months fresh) was collected from local feedlots and analyzed for total N using a Leco FP-528 nitrogen/protein Determinator (St. Joseph, MI). Manure application rate was calculated by converting total N kg\(^{-1}\) manure to the specified rate (56 kg N ha\(^{-1}\) for grasses, and 11.2 kg N ha\(^{-1}\) for legumes) for 5m\(^2\) plots with an assumed N loss of 40% over the season and 25% volatilization loss immediately after application (Beauchamp and Paul, 1989; Chambers et al., 1997; Chambers et al., 1999). We assumed the impact of other nutrients in manure to be minimal in this short-term study. Urea was used for our synthetically fertilized treatment, and both urea and manure were surface applied. All fertilized plots were irrigated (4-cm water) immediately after urea or manure application to minimize N loss through volatilization.
Sample Collection and Analysis

In each treatment (synthetic fertilizer, feces, and control) from each plant species (Alf, E+TF, RCG, and BFT) multiple subsamples (n=10) were clipped at 5 cm above ground then composited. Samples from each forage were selected in a simple random approach three times in 2007 (20 Aug., 13 Sept., and 15 Oct.), and four times in 2008 (2 June, 2 July, 1 Aug., and 16 Sept.) when developmental morphology (late vegetation) was similar at all collection times for each species (Flick and Mueller, 1989; Moore et al., 1991; Moore and Moser, 1995). After each sampling period plots were mowed and plant matter removed, then regrowth was allowed between sampling periods. Plant samples were placed on dry ice in the field, then stored at -20°C until freeze-dried. Dried plant samples were ground to pass a 1-mm screen with a Wiley mill grinder (Thomas Scientific, Swedesboro, NJ), then stored in sealed plastic bags at -20°C until chemical analyses. Each plant species included 63 composite samples for analysis (3 treatments x 7 collection periods x 3 blocks).

Two subsamples were taken from each composite plant sample for independent analysis for total N using a Leco FP-528 nitrogen/protein Determinator (St. Joseph, MI), and a mean value was computed over the two subsamples.

Alfalfa samples were analyzed for total saponin concentration using a modification (Appendix A) of Lee et al. (2001) using methods developed by Patamalai et al. (1990) and Wall et al. (1952). Each ground Alf sample was weighed (50 mg) in triplicates in 16 mL screwcap test tubes. Subsamples were extracted with MeOH (methanol, 5 mL) by inverting (30 min), then centrifuging (5 min) (2500 rpm) (2X). Both MeOH extracts were combined and solvent was allowed to evaporate under fume hood to
dryness. Residue was extracted using 5:5 CHCl₃ (chloroform):distilled water mix (10 mL) (1X) and CHCl₃ (5 mL) (3X), discarding CHCl₃ layer each time. The aqueous extract was then extracted with water-saturated C₄H₁₀O (n-butanol, 5 mL) (3X). The C₄H₁₀O extracts were combined and solvent removed by drying under fume hood. Distilled water (5 mL) was added to each dried sample and underwent a vigorous two-minute shake. Foam was then measured to determine percent by weight saponin content of each sample based on a five-point standard curve (R² = 0.98).

Birdsfoot trefoil samples were analyzed for CT as described by Mantz et al. (2008) using methods developed by Terrill et al. (1992) and Reed (1986). Each ground BFT sample was weighed (500 mg) in triplicates in 50 mL centrifuge tubes. Subsamples were extracted with a 7:3 C₃H₆O (acetone):distilled water mixture (20 mL) by vortexing (10 sec) (2X) with Vortex-Genie 2 (Bohemia, NY), then placed in Shel Lab shaking water bath (Baltimore, MD) (32°C, 95 RPM) (15 min), and then centrifuged (2500 rpm) (15 min) (3X). All three C₃H₆O extracts were combined and solvent (100 µL) was placed in 16 mL screwcap test tubes in triplicates with a 95:5 C₄H₁₀O:HCl (hydrochloric acid - 37%) mixture (5 mL), vortexed with Vortex-Genie 2 (10 sec), capped with glass marbles, then placed in water bath (95°C) (75 min), and then tubes were cooled in ice water. Samples were measured using Unico Spectrophotometer (Holbrook, NY) (550 nm).

Endophyte-infected tall fescue samples were analyzed for ergovaline using HPLC methods developed by Rottinghaus et al. (1991) and Hill et al. (1993). Each ground E+TF sample was weighed (5 g) and put into polypropylene wide mouth screwcap bottles with CHCl₃ (100 mL) and 0.1 M NaOH (sodium hydroxide, 5 mL). The samples were then mixed by mechanical rotation for >16 hrs (overnight). Sodium sulfate (2 g) was added to
each sample, then samples were placed on shaker (30 min). Extracts (~20 mL) were filtered through Whatman PS-1 filter paper, then extract (10 mL) was applied to an Ergosil cleanup column (Analtech/ iChromatography, Newark, DE) under vacuum. Pigments were eluted from the column with an 8:2 C₃H₆O:CHCl₃ mixture (1.8 mL) followed by anhydrous petroleum ether (3 mL). Ergopeptine alkaloids were then eluted with MeOH into a 2 mL volumetric tube. The MeOH eluant was then passed through a small M-224 cleanup column (Romer Labs, Getzersdorf, Austria) to remove the last remaining pigmentation prior to HPLC analysis.

Analysis of the alkaloid gramine was preformed using a modification of Anderton et al. (1999). Each ground RCG sample (100 mg) was weighed and placed into 15 mL screw cap test tubes. Dilute HCl (1N, 4 mL) and CHCl₃ (4 mL) were added to the tubes and sealed with Teflon lined caps. The samples were then mixed by mechanical rotation for >16 hrs (overnight). The samples were centrifuged to aid layer separation. The upper acid layer was removed and added to a prewashed (2 mL MeOH, 2 mL deionized water) solid phase extraction (SPE) column (Phenomenex Strata –X-C polymeric strong cation, part # 8B-S029-UBJ). The acid solution was pulled through column under vacuum, the column then rinsed with 2 mL 0.1 N HCl and then allowed to dry under vacuum for one minute. The SPE column was rinsed with 2 mL MeOH and then the analyte removed by elution with 4 mL 5% ammonium hydroxide in MeOH into clean 8 mL screw cap vials. The solvent was then evaporated under a flow of N with the samples in a heated (60°C) holding block. The samples were then dissolved in 1.0 mL of 50% MeOH (0.1% trifluoroacetic acid), mixed and then transferred to LC autosample vials. A standard stock solution of gramine was prepared (1.0 mg mL⁻¹) in MeOH. Calibration standards were
then prepared by dilution of 0.200 mL of stock with 1.8 mL 50% MeOH (0.1% TFA),
then serial diluted to make standards at (50, 25, 12.5, 6.25, and 3.13 ppm). Samples and
gramine calibration standards were then analyzed by high performance liquid
chromatography-mass spectrometry (HPLC-MS) using a Thermo Electron LCQ
Advantage Max mass spectrometer and a Thermo Electron Sureyor autosampler and MS
pump. The HPLC column was an Aquasil C18 (100 x 2.1 mm) eluted with a gradient
flow of 0.1% TFA and acetonitrile (ACN); 10% ACN (0-3 min.); 10% - 70% ACN linear
increase (3 – 10 min); 70% ACN (10-15 min). Column flow was connected directly to an
atmospheric pressure chemical ionization (APCI) source with a mass spectrometer
scanning a mass range of 70-800 m/z. HPLC peak areas were integrated from
reconstructed ion chromatograms using m/z 130 (MH⁺, gramine).

**Statistical Analyses**

Each forage was analyzed separately using a RCBD with three replications, using
a 1-way treatment structure (synthetic fertilizer, feces, and control) with collection period
repeated measures. The response variables were saponins (g kg⁻¹), ergovaline (µg kg⁻¹),
gramine (mg kg⁻¹), condensed tannins (g kg⁻¹), and N (g kg⁻¹). Analyses were conducted
using SAS PROC GLIMMIX (SAS Institute, Cary, NC). Differences between LS means
were adjusted for family-wise Type I error using the Tukey-Kramer method.
RESULTS

Alfalfa with Feces or Synthetic Fertilizer

No differences (P = 0.76, SE = 1.79) in saponin concentration were found when Alf was fertilized with feces (13.7 g kg\(^{-1}\)), urea (synthetic fertilizer, 14.5 g kg\(^{-1}\)), or unfertilized (13.9 g kg\(^{-1}\)) yet saponin concentration and total N changed significantly over time (P < 0.0001; Fig. 3-1). Total N treatment means in Alf tended to be greater (P = 0.08; SE = 0.35) in synthetically fertilized plots (44.5 g kg\(^{-1}\)) than in plots fertilized with feces (43.6 g kg\(^{-1}\)) or without fertilizer (43.3 g kg\(^{-1}\)).

![Graph showing saponin, Alf N, Tannins, and BFT N concentrations over time from Aug. 2007 to Sept. 2008.]

**Fig. 3-1.** Saponin means in alfalfa (g kg\(^{-1}\)) over time (P < 10\(^{-4}\)), tannin means in birdsfoot trefoil (g kg\(^{-1}\)) over time (P = 0.003), and total N means in Alf and BFT (g kg\(^{-1}\)) over time (both P < 10\(^{-4}\)) are shown (and SE) for 7 collection periods from 2007-2008.

Birdsfoot Trefoil with Feces or Synthetic Fertilizer

No differences (P = 0.45; SE = 4.92) in CT concentration were found when BFT was fertilized with feces (20.6 g kg\(^{-1}\)), urea (28.6 g kg\(^{-1}\)), or unfertilized (28.9 g kg\(^{-1}\)).
Over time, CT \((P = 0.003)\) and N means \((P < 0.0001)\) in BFT differed significantly (Fig. 3-1). No differences \((P = 0.49; SE = 0.82)\) in total N were observed when BFT was fertilized with feces \((36.7 \text{ g kg}^{-1})\), urea \((36.7 \text{ g kg}^{-1})\), or unfertilized \((36.1 \text{ g kg}^{-1})\).

**Endophyte-Infected Tall Fescue with Feces or Synthetic Fertilizer**

No differences \((P = 0.15; SE = 23.6)\) in ergovaline concentration were observed when E+TF was fertilized with feces \((249 \mu\text{g kg}^{-1})\), urea \((297 \mu\text{g kg}^{-1})\), or unfertilized \((222 \mu\text{g kg}^{-1})\). Ergovaline concentration and total N in E+TF differed over time \((P < 0.0001; \text{Fig. 3-2})\). Total N means in E+TF were greater \((P = 0.005; SE = 0.92)\) in plots fertilized with urea \((33.4 \text{ g kg}^{-1})\) than in plots fertilized with feces \((28.5 \text{ g kg}^{-1})\) or unfertilized plots \((29.2 \text{ g kg}^{-1}; \text{Fig. 3-2})\).

![Graph showing ergovaline and N means over time](image)

**Fig. 3-2.** Ergovaline means \((\mu\text{g kg}^{-1})\) in endophyte-infected tall fescue are shown over time \((P < 10^{-4})\). Total N \((\text{g kg}^{-1})\) treatment means \((P = 0.005)\) in endophyte-infected tall fescue fertilized with feces (fecal), urea (synthetic), and without fertilizer (control) are shown over time \((P < 10^{-4})\) for 7 collection periods from 2007-2008.
Reed Canarygrass with Feces or Synthetic Fertilizer

Gramine means in RCG tended (P = 0.062; SE = 122.9) to be greater in plots without fertilizer (1223 mg kg\(^{-1}\)) than in plots fertilized with urea (1119 mg kg\(^{-1}\)) or feces (1013 mg kg\(^{-1}\)). Gramine and total N means in RCG differed over time (P < 0.0001; Fig. 3-3). Total N means in RCG were greater (P = 0.004; SE = 1.06) in synthetically fertilized plots (36.8 g kg\(^{-1}\)) than in unfertilized plots (32.1 g kg\(^{-1}\)) or in plots fertilized with feces (31.0 g kg\(^{-1}\); Fig. 3-3).

![Fig. 3-3. Gramine means (mg kg\(^{-1}\)) in reed canarygrass are shown over time (P < 10\(^{-4}\)). Total N (g kg\(^{-1}\)) treatment means (P = 0.004) in reed canarygrass fertilized with feces (fecal), urea (synthetic), and without fertilizer (control) are shown over time (P < 10\(^{-4}\)) for 7 collection periods from 2007-2008.](image)

DISCUSSION

We determined how different N sources (urea or feces) influence plant secondary metabolites and N concentrations during the growing season for Alf, E+TF, RCG, and BFT. We hypothesized that concentrations of N-based PSM like alkaloids, i.e., gramine
and ergovaline in RCG and E+TF, respectively, would be greater in plots fertilized with urea initially, with a delayed increase for plots fertilized with feces as nutrients were released from that source at a slower rate. Contrary to our hypothesis and other studies (Rottinghaus et al., 1991; Arechavaleta et al., 1992), ergovaline concentration in E+TF was not affected by fertilization. Moreover, gramine content in RCG was greater in control plots than in fertilized plots. Fertilization and irrigation that rapidly increase yield can cause nutrient declines of at least some nutrients due to the “dilution effect” (Jarrell and Beverly, 1981), a pattern which could explain the lower than expected concentration of ergovaline in fertilized plots. Alternatively, our fertilization rates (168 kg ha⁻¹) were likely not high enough to render a response in alkaloid production by plant tissues (Marten, 1973).

Consistent with our hypotheses, total N in both E+TF and RCG was greater in synthetically fertilized plots than in feces-fertilized or unfertilized plots. Interestingly, N concentration in E+TF was greatest in June, 2008 while ergovaline amounts were at the lowest values recorded, with subsequent increases to the greatest ergovaline values observed in July, 2008. Prior research on tall fescue suggests greater N concentration renders greater fungal-derived (Rottinghaus et al., 1991; Palumbo et al., 2007) and plant-derived (Gentry et al., 1969) alkaloid concentrations, likely driven by simple mass action. In separate studies at the same study site, Clemensen et al. (2018) and Friend et al. (2015) found a positive correlation between total N and ergovaline in E+TF when the plant grew adjacent to N-fixing legumes.

We cannot explain the temporal disjoint in ergovaline and total N content in E+TF between June and July, 2008. However, as Neotyphodium coenophialum exhibits
mutualism with tall fescue (Hill et al., 1990; 1991) and rhizobia shows a mutualistic association with legumes (Bottomley and Myrold, 2007) grasses and legumes growing adjacentely also show symbiotic interaction (Haynes, 1980; Mallarino et al., 1990; Danso et al., 1991). We can only speculate that the consistent source of N to E+TF via N-fixing legumes versus sudden bursts of N availability from fertilizers affects the allocation of N to the production of ergovaline.

We also hypothesized that C-based PSM like saponins in Alf or CT in BFT would be greater in plots fertilized with feces due to mass action driven by greater carbon availability to the plant. However, there were no differences between treatments for Alf or BFT PSM. These results may be due to the minimal amount of fertilizer, as recommended (Koenig et al., 1999; Koenig et al., 2002), which was applied to the legumes in the present study. In their field study Hofland-Zijlstra and Berendse (2009) found N fertilizer (NH₄NO₃) decreased total phenolics in ericaceous plants without affecting CT concentrations. In contrast, sapogenin concentrations in Asparagus racemosus increased with N, as well as with phosphorus and potassium fertilizers (Vijay et al., 2009). In water-stressed Bupleurum chinense DC. plants, saponin contents appeared greater in fertilized treatments compared to unfertilized plants, yet saponin concentration in the roots were lower with larger amounts of fertilizer (Zhu et al., 2009). Thus, type and concentration of fertilizer, as well as plant part all have an influence on concentrations of saponins and CT. We measured C-based PSM concentrations in the whole shoot systems of Alf and BFT. Differences in concentrations in different plant tissues were likely diluted with this approach.
Our study, in addition to prior research, suggests that seasonal variation is highly influential in PSM production. In some circumstances saponins in Alf, as well as CT in BFT, change with season; being greater in the summer and lower in the spring and fall (Cheeke, 1998; Tava et al., 1999). Studies have shown greater alkaloid levels may be a result of high temperatures, moisture stress, low light, as well as immature tissues (Marten, 1973; Majak et al., 1979; Thompson et al., 2001). While endophyte frequency and concentration may be greater in months with warmer temperatures and less precipitation, it is possible that the endophyte response is related to vernalization and physiological or morphological changes occurring in the plants (Ju et al., 2006).

In summary, we found the most influential factor influencing the concentration of PSM in Alf, E+TF, BFT, and RCG was season. The most substantial differences between fertilization treatments were in RCG where gramine contents decreased with fertilization. We also found greater total N content in all forages fertilized with urea, except for BFT. Our research is limited in that we did not determine physiological reasons behind plant resource allocation as our experiments did not explore physiological mechanisms in the plant. Our results exemplify the inconsistencies in PSM production in plants and highlight the complexities of predicting fluctuations of PSM in forages. As Hartmann (2007) acknowledges of plant intricacies, “primary metabolism covers all processes essential for growth and development whereas secondary metabolism is indispensable for the survival of the individual in its environment.” As environments where plants grow vary through space and time, we recommend studies on a case-by-case basis depending on land management objectives.
REFERENCES


CHAPTER 4
CAN CONDENSED TANNINS AND SAPONINS REDUCE NITROGEN LOSS IN PASTURE AGROECOSYSTEMS BY REDUCING NITROGEN MINERALIZATION?

Abstract

Pasture and agricultural systems are susceptible to rapid mineralization rates with ensuing manure additions, rendering substantial amounts of nitrogen (N) loss which increases eutrophication and ultimately affects the global N cycle. Plant secondary metabolites (PSM) influence soil dynamics in boreal forest systems by slowing mineralization rates. We investigated whether cattle-grazed pasture legumes such as sainfoin (*Onobrychis viciifolia* Scop.) or alfalfa (*Medicago sativa* L.) in monocultures (Mono) and two-way grass (*Festuca arundinacea* Schreb.) legume mixtures (Mix) produce sufficient amounts of condensed tannins (CT) or saponins, respectively, to influence soil nutrient cycling. Throughout the study, CT in sainfoin averaged 58.9 g kg\(^{-1}\) whereas saponins in alfalfa averaged 5.7 g kg\(^{-1}\). We found greater (*p* = 0.02) soil nitrate (NO\(_3^-\)) in alfalfa than in sainfoin plots, yet total differences in N content between legumes were minimal. We observed greater soil microbial respiration (*p* = 0.005) and dehydrogenase enzyme activity (*p* = 0.05; DHEA) in grass Mono, indicating both legumes and inherent PSM may inhibit soil microbial and enzymatic activity. We also conducted a laboratory soil-feces incubation study to determine if cattle feces at high- and low- rates from differing diets of CT-containing sainfoin or saponin-containing alfalfa influence soil nutrient cycling. We found that application of cattle feces at high rates decreased N
mineralization, with greater \( p < 0.0001 \) NO\(_3\)^– in the low-rate treatment. Our data suggests PSM in pasture forages may influence soil nutrient cycling. We propose that implementing tanniferous and/or saponin-containing forages in pasture agroecosystems may decrease N mineralization, thus reducing N loss.

1. Introduction

Plant secondary metabolites (PSM), such as condensed tannins (CT), benefit animal agricultural ecosystems from the atmosphere to the soil. Previous research suggests tannin-containing forages may reduce methane emissions from grazing animals (Pinares-Patiño et al., 2003; Woodward et al., 2004a, 2004b; Boadi et al., 2004; but see Beauchemin et al., 2007). On the other hand, animals grazing an array of diverse forages, including tanniferous forages, improves food production while enhancing soil quality and nutrient cycling (Tracy et al., 2018). Research suggests diverse patchworks of vegetation containing PSM increase rate of gain in foraging animals (e.g. Meuret and Provenza, 2015) while increasing the resilience of agroecosystems (Tracy et al., 2018) through mechanisms that span from complementarities among resources to nutrient-cycling feedbacks, which increase nutrient stores in soils (Tilman et al., 2014; Tilman and Snell-Rood, 2014). Nevertheless, nitrogen (N) loss in agroecosystems is widespread, and the potential for nitrate (NO\(_3\)^–) leaching under grazed pastures is greater than that of mowed pastures, as 60-90% of the ingested N is returned to the soil via manure (Haynes and Williams, 1993; Di and Cameron, 2002). The presence of condensed tannins in plant tissues may ameliorate this problem as these PSM bind to proteins in the gastrointestinal tract, increasing the fecal:urinary N ratio (Barry and McNabb, 1999; Waghorn et al.,
1994) which ultimately slows the release and leaching potential of N in pasture agroecosystems (Powell et al., 2009).

Condensed tannins are large polar molecules that when consumed by grazing animals remain in the gastrointestinal tract and are excreted with the feces (Waghorn, 2008). In addition to condensed tannins, terpenes (i.e. saponins) are known to affect both carbon (C) and N cycling in the soil (Smolander et al., 2012), particularly by increasing N immobilization (Bradley et al., 2000). The mechanisms of how plant tannins affect soil N cycling have been investigated, particularly in boreal forest systems, and indicate that these PSM may bind organic N compounds, representing an increased C resource for soil microbes or becoming toxic to microbes, although their specific roles are not fully understood (see Smolander et al., 2012). Research in silvicultural soil systems shows PSM, such as CT and terpenes, influence C and N cycling, decrease decomposition rates, and inhibit soil mesofauna and soil enzyme activity (B. Adamczyk et al., 2011, 2009; S. Adamczyk, 2013; Bradley et al., 2000; Joanisse et al., 2007; Lorenz et al., 2000; Madritch and Lindroth, 2015; Smolander et al., 2012). Phenolic compounds (i.e. tannins) are known to hinder nitrification in soil by inhibiting N-fixing bacteria such as Nitrosomonas europaea, and the rate at which plants exude these inhibiting compounds depends on soil pH and plant growth stage (AlSaadawi, 1988; Subbarao et al., 2007; Zakir et al., 2008). Kelleher et al. (2006) describe research supporting the idea that CT are either adsorbed to soil minerals, bound to other proteinaceous substances, or transformed.

Soil research involving PSM has increased over the past decade, particularly in silviculture. The only research we are aware of regarding the influence of PSM on
pasture soils is with ergot alkaloids (Franzluebbers et al., 1999; Franzluebbers, 2006; Franzluebbers and Hill, 2005; Franzluebbers and Stuedemann, 2005; Omacini et al., 2004). To our knowledge there is no research regarding effects of CT or saponins in pasture agricultural soils, or if pasture forages produce enough of these metabolites to influence soil nutrient dynamics. The tannin content of detritus in forest systems is much greater than that present in other ecosystems; CT are not easily decomposed, and they heavily influence N and C mineralization (Kraus et al., 2003). Research suggests C-based compounds such as tannins and terpenes inhibit N mineralization thereby increasing N immobilization, and the effects of CT on this process are generally greater than those observed for terpenoids due to their greater reactivity with N (S. Adamczyk et al., 2013; Barry et al., 1986; Smolander et al., 2012; Winder et al. 2013). Collectively, nitrogen immobilization would benefit pasture agricultural systems by reducing N loss via leaching, so we explored whether the same phenomenon in silvicultural soil may occur in pasture agricultural soil with PSM-containing forages.

We investigated how cattle grazed pastures of two different N-fixing PSM-producing legumes; (i) CT-containing sainfoin (Onobrychis viciifolia Scop.), or (ii) saponin-containing alfalfa (Medicago sativa L.), with novel-endophyte tall fescue grass (Festuca arundinacea Schreb.; TF) in monocultures (Mono) and two-way grass/legume mixtures (Mix) affect soil quality and nutrient cycling. We addressed whether CT from sainfoin or saponins from alfalfa are produced in sufficient amounts to affect soil nutrient cycling in pasture agricultural systems by (i) measuring the concentration of both CT and saponins in plant samples, and (ii) measuring soil inorganic N, enzymatic activity, and other soil responses. We hypothesized that (i) grazed TF plots would have greater
evidence of soil immobilization, while grazed legume plots would show increased mineralization rates, and (ii) CT-containing sainfoin plots would show more signs of increased N-immobilization than saponin-containing alfalfa plots given the high reactivity of CT with N. To illuminate the effects of manure on soil nutrient cycling we also performed a laboratory soil incubation study with cattle feces from a field experiment with two different diets consisting of TF mixed with either CT-containing sainfoin or saponin-containing alfalfa.

2. Materials and methods

2.1. Field experiment - plot establishment

Research plots were established in spring 2009 at Utah State University’s Agriculture Research Field Station in Lewiston, Utah at 41° 57' 4" N, 111° 52' 26" W. The study site consisted of (i) Kidman fine sandy loam (coarse-loamy, mixed, mesic Calcic Haploxeroll) and (ii) Lewiston fine sandy loam (coarse-loamy, mixed, mesic Aeric Calciaquoll). The field experiment simulated a whole plot split of legume (alfalfa and sainfoin), and a second split factor of planting configuration (legume Monoculture – Legume Mono, Tall fescue monoculture – TF Mono, and grass/legume Mix) in three blocks. Planting configuration strips were 125 x 30.5 m. Monoculture strips were seeded with novel-endophyte TF variety ‘Max Q’ at 28 kg ha\(^{-1}\), inoculated alfalfa variety ‘Vernal’ at 11 kg ha\(^{-1}\), and inoculated sainfoin variety ‘Shoshone’ at 33 kg ha\(^{-1}\). The grass/legume Mix (e.g., TF/alfalfa and TF/sainfoin) was seeded at a 30:70 (grass: legume) rate. Angus fall-born calves strip-grazed pastures from May-September 2010,
and June-September 2012, with temporary electric fence, allowing for daily fresh forage access (Maughan et al., 2014).

After forages were grazed in 2010, Mix plots became predominantly TF, as the TF outgrew each legume. Grazing proceeded through September 2010 while plant collections ceased, with attempts to reestablish Mix treatments by spreading more inoculated seed of both legume species in their respective plots. However, both legumes in Mix treatments failed to reestablish. Therefore TF and legume samples for Mix treatments were collected along the boundary of TF and either alfalfa or sainfoin strips, where TF and either legume were growing directly adjacent to each other. Due to overwinter crop failure (2010-2011 winter) alfalfa and sainfoin Mono plots over all 3 blocks required reseeding in spring 2011, therefore the project resumed in 2012. From June – September 2011 treatments were swathed and baled.

2.1.2. Soil Sample Collection and Analysis

Before seeding in summer (July 6-7) 2009, baseline soil subsamples (n=10) were collected, then composited, in a zigzag pattern in each strip using a Giddings probe to a depth of 90 cm and split into three increments (0-30, 30-60, and 60-90 cm). Soil samples were sieved to pass a 2-mm screen and stored at 4°C until analysis within 2 weeks. Baseline soil samples were analyzed (Gavlak et al., 2003) for nitrate-nitrogen (NO₃⁻ -N), using the Cadmium Reduction method (Method S-3.10), and ammonium (NH₄⁺ -N; Method S-3.50) at 0-30, 30-60, and 60-90 cm increments. A subset (0-30 cm depth) was analyzed for dehydrogenase enzyme activity (DHEA) using methods described by Tabatabai (1994). Soil samples were then air-dried and analyzed (Gavlak et al., 2003) for
soil pH (Method S-2.20), electrical conductivity (EC; Method S-2.30), Olsen extractable P and K (Method S-4.10), and DTPA-extractable Fe, Zn, Cu, and Mn (Method S-6.10). Total and organic carbon was analyzed using a Skalar Primacs SLC model CS22 (Breda, Netherlands), using the two temperature (575 - 1035°C) method described by Chichester and Chaison (1992). Total N was analyzed using a Skalar Primacs Solid Sample TN Analyzer (Breda, Netherlands). Baseline soil samples included 18 composite samples for analysis (2 legumes x 3 planting configurations x 3 blocks). Baseline soil samples taken in 2009 before plot establishment showed no differences among response variables between experimental plots.

In spring (May 2-5) 2011 soil samples were collected to determine treatment effects two years after pasture establishment. Soil subsamples (n=10) were collected, then composited, in a zigzag pattern in each plot using a soil probe to a depth of 30 cm. Soil samples were sieved to pass a 2-mm screen and stored at 4°C until analysis within 2 weeks. Soil samples were analyzed for NO$_3^-$ -N, NH$_4^+$ -N, and the top 10 cm increments analyzed for DHEA, as described above.

In fall (October 9-19) 2012 end-of-study soil samples were collected. Soil subsamples (n=5) were collected in a zigzag pattern in each plot using a Giddings probe to a depth of 90 cm, and split into five increments (0-10, 10-20, 20-30, 30-60, and 60-90 cm), each subsample composited for all five increments. Soil samples were sieved to pass a 2-mm screen and stored at 4°C until analysis. Soil samples from 2012 were analyzed for NO$_3^-$ -N and NH$_4^+$ -N at 0-30, 30-60, and 60-90 cm increments, and a subset (0-30 cm) was analyzed for pH, EC, total N, total and organic C, P, K, Zn, Fe, Cu, and Mn as described above. DHEA was analyzed at 0-10, 10-20, and 20-30 cm increments. A subset
(0-10 cm) was analyzed for phenol oxidase (Prosser et al., 2011) and soil respiration, with ratios between microbial biomass to organic carbon calculated to determine metabolic efficiencies (Anderson and Domsch, 1978; Davidson et al., 1987; Smith et al., 1985; Sparling, 1992). In November 2012 soil bulk density was measured, and soil porosity calculated, using methods developed by Blake (1965) and described by USDA ARS NRCS Soil Quality Institute (2001). Soil samples included 18 composite samples for analysis from each depth.

2.1.3. Plant Sample Collection and Analysis

In spring (May 14-19) 2010, before treatments commenced, baseline plant samples from each plant species (alfalfa, sainfoin, and TF) were collected in a simple random approach in each treatment. Subsamples of each species (n=10) were clipped at 5 cm above ground then composited. Plant samples were gathered when developmental morphology was similar for each species, at late vegetative growth (Flick and Mueller, 1989; Moore et al., 1991; Moore and Moser, 1995). Plant samples included twelve composite TF samples (2 legumes x 2 planting configurations x 3 blocks), six composite alfalfa samples, and six composite sainfoin samples (2 planting configurations x 3 blocks). Sainfoin samples were placed on dry ice in the field, then stored at -20°C until freeze-dried. Alfalfa and TF samples were placed in drying ovens at 30°C. Dried plant samples were ground to pass a 1-mm screen with a Wiley mill grinder (Thomas Scientific, Swedesboro, NJ), then stored in sealed plastic bags at -20°C until chemical analyses.
In 2012 plant samples were collected 3 times (26 June, 14 Aug., and 18 Sept.) from each plant species as described above. Plant samples from each species from each of the two grass/legume Mix areas were collected in separate bags. After sampling periods, plots were grazed, then regrowth was allowed between collections. Plant samples included 36 composite TF samples (2 legumes x 2 planting configurations x 3 collection times x 3 blocks), 18 composite alfalfa samples, and 18 composite sainfoin samples (2 planting configurations x 3 collection times x 3 blocks). Plant biomass was collected throughout the experiment and detailed in Maughan et al. (2014).

Each composite plant sample was analyzed for total N using a Skalar Primacs Solid Sample TN Analyzer (Breda, Netherlands). Sainfoin samples were analyzed for CT as described by Mantz et al. (2008) and Clemensen et al. (2017) using methods developed by Reed (1986). Alfalfa samples were analyzed using a modification of Lee et al. (2001) as described by Clemensen et al. (2017) using methods developed by Patamalai et al. (1990) and Wall et al. (1952).

2.1.4. Statistical Analyses

The field experiment employed a split-plot design with three replicates in complete blocks (RCBD). Legume (alfalfa or sainfoin) was the whole plot factor and planting configuration (legume Mono, TF Mono, and Mix) was the subplot factor. Soil analyses included 5 depths (0-10, 10-20, 20-30, 30-60, and 60-90 cm); depth was assessed separately. Soil data for each year (2009, 2011, and 2012) was analyzed separately. Plant data includes repeated measures with three months of data collections (June, July, and August) over two years (2010 and 2012), each year was analyzed
separately. Analyses were conducted using SAS PROC GLIMMIX (SAS Institute, Cary, NC). Differences between least square means were adjusted for family-wise Type 1 error using the Tukey-Kramer method.

2.2. Laboratory incubation study – soil and feces collection and analysis

Soil was collected from the top 30 cm in the grass Mono treatment from the field experiment site and air dried, then sieved through a 2 mm screen. Fresh cattle feces was collected from aforementioned grazing experiments where Angus fall-born calves grazed on pastures of either inoculated alfalfa variety ‘Vernal’, or inoculated sainfoin variety ‘Shoshone’, both of which were in monocultures with adjacent monocultures of novel-endophyte tall fescue variety ‘Max Q’. Tannin content in sainfoin averaged 58.9 g kg\(^{-1}\) whereas saponins in alfalfa averaged 5.7 g kg\(^{-1}\) during the grazing period.

Fresh feces from each ‘diet’ (CT-containing sainfoin or saponin-containing alfalfa) was collected fresh and analyzed for pH as described above, and total N and C using Leco FP-528 total combustion (St. Joseph, MI). Feces from alfalfa diets had 7.28 pH, 3.0 g N kg\(^{-1}\), and 47.8 g C kg\(^{-1}\). Feces from sainfoin diets had 7.26 pH, 3.6 g N kg\(^{-1}\), and 44.8 g C kg\(^{-1}\). Fecal samples were then stored at -20°C, until freeze dried. Cattle feces was then ground and sieved through a 2 mm screen to be used in the soil incubation experiment.

2.2.1. Laboratory Incubation Experimental Design and Analysis

The incubation study employed a block design with two factors with repeated measures, where feces from either alfalfa or sainfoin-based diets were mixed with soil at two different rates and incubated at 24°C for 56 days. Each treatment had four replicates
containing 500 g air-dried soil. Feces rates were based on; (i) the assumption of daily excretions (Nennich et al., 2005), our low-rate treatment, and (ii) N fertilizer recommendations from N content of feces (Honeycutt et al., 2005), our high-rate treatment. Assumed excretions for the Angus fall-born calves were 3.74 kg DM day⁻¹, and calculations determining the amount of feces to be added to each subsample of soil (Honeycutt et al., 2005) used a bulk density of 1.3 g cm⁻³.

On day 0, distilled water was added to each subsample of soil and feces to bring it to 18% moisture by slowly misting while mixing, then each subsample was slightly compacted to reach a bulk density of roughly 1.3 g cm⁻³. The initial weight of all samples was recorded and moisture content monitored, adding distilled water when evaporation loss was > 5% (relative) of 18% moisture. Samples were placed in sealed quart-size Ziploc® bags with protruding straws to allow exchange of gases and minimize water loss, and stored at 24°C (Baitilwake et al., 2012; Honeycutt et al., 2005).

Sampling for analyses occurred at days 0, 3, 7, 14, 21, 28, 42, and 56. Samples were analyzed for NO₃⁻, NH₄⁺, DHEA, organic C, and total N as described in section 2.1.2. Total N and organic C was measured at the beginning and end of the incubation period (days 0 and 56). Two feces types, both at low- and high-rates, including the control, totaled 160 samples for analysis (5 treatments x 4 replications x 8 sample days).
2.2.2. Calculations

Feces rate based on daily excretions:

Equation 1:

\[
\frac{3.74 \text{ kg DM manure}}{\text{cow per day}} \times \frac{12 \text{ cows}}{12,285.75 \text{ m}^2} \times \frac{10,000 \text{ m}^2}{\text{ha}} \times \frac{81 \text{ days}}{1} = \frac{2959.18 \text{ kg DM manure}}{\text{ha}}
\]

Equation 2:

\[
\frac{2959.18 \text{ kg DM manure}}{\text{ha}} \times \frac{\text{ha furrow slice}}{1500 \text{ m}^3} \times \frac{\text{m}^3}{1,000,000 \text{ cm}^3} \times \frac{\text{cm}^3}{1.3 \text{ g soil}} \times \frac{1000 \text{ g}}{\text{kg}} \times \frac{500 \text{ g soil}}{\text{bag}} = \frac{0.76 \text{ g manure}}{\text{bag}}
\]

Feces rate based on N content:

Equation 1:

\[
\frac{350 \text{ kg N}}{\text{haf s}} \times \frac{\text{haf s}}{1500 \text{ m}^3} \times \frac{\text{m}^3}{1,000,000 \text{ cm}^3} \times \frac{\text{cm}^3}{1.3 \text{ g soil}} \times \frac{1000 \text{ g}}{\text{kg}} \times \frac{100 \text{ kg DM}}{2.14 \text{ kg N}} \times \frac{500 \text{ kg soil}}{\text{bag}} = \frac{4.19 \text{ g DM manure}}{\text{bag}}
\]

2.2.2. Statistical Analysis

The laboratory incubation experiment employed a block design with two factors (diet and feces rate) with repeated measures (days 0, 3, 7, 14, 21, 28, 42, and 56).

Analyses were conducted using SAS PROC GLIMMIX (SAS Institute, Cary, NC).

Differences between least square means were adjusted for family-wise Type 1 error using the Tukey-Kramer method.
3. Results and discussion

3.1. Field experiment

3.1.1. Legume Species - Effects on Soil

To determine how saponin-containing alfalfa or CT-containing sainfoin influenced soil nutrient cycling we measured soil responses under each legume treatment from the field experiment. We hypothesized that between legumes, CT from sainfoin would inhibit soil N mineralization to a greater extent than saponins from alfalfa. Consistent with our hypothesis, soil NO$_3^-$ concentration was 3.2-fold greater ($p = 0.02$) in alfalfa than in sainfoin plots at 0-30 cm depth in the fall of 2012 (Table 4-1), indicating increased mineralization in alfalfa plots. Alfalfa plots also showed greater EC than sainfoin plots ($p = 0.04$), possibly explained by the greater soil NO$_3^-$ content in this treatment. Both sainfoin and alfalfa fix N efficiently, yet Krall and Delaney (1982) found sainfoin was more proficient at N-fixation. Our results clearly show greater NO$_3^-$ content in alfalfa plots in 2012 which suggests CT from sainfoin reduce N mineralization, consistent with other studies in boreal forest systems (Smolander et al., 2012). However, plant productivity data (Maughan et al., 2014; Table 4-2) at the time of the 2012 soil collection (September) shows values for alfalfa biomass (42 t ha$^{-1}$) substantially greater than those observed for sainfoin (27 t ha$^{-1}$), which may also explain the differences in NO$_3^-$ concentration between the two legumes.
Table 4-1
Field experiment 2012 soil results with standard errors (SE) and, where applicable, letter depicting statistical differences between tall fescue monocultures (TF Mono), legume monocultures (Legume Mono), and grass/legume two-way mixtures (Mix).

<table>
<thead>
<tr>
<th>Soil property</th>
<th>Legume (SE)</th>
<th>Planting Configuration (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alfalfa</td>
<td>Sainfoin</td>
</tr>
<tr>
<td></td>
<td>TF Mono</td>
<td>Legume Mono</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>g⁻¹ soil</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium, µg (0-30 cm depth)</td>
<td>0.48 (0.47)</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>Ammonium, µg (30-60 cm depth)</td>
<td>0.00 (0.38)</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Ammonium, µg (60-90 cm depth)</td>
<td>0.00 (0.20)</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Nitrate, µg (0-30 cm depth)</td>
<td>7.47 (0.53) *</td>
<td>1.76</td>
<td></td>
</tr>
<tr>
<td>Nitrate, µg (30-60 cm depth)</td>
<td>1.26 (0.36)</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>Nitrate, µg (60-90 cm depth)</td>
<td>0.50 (0.19)</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Dehydrogenase, µg TPF (0-10 cm depth)</td>
<td>9.05 (0.74)</td>
<td>10.7</td>
<td></td>
</tr>
<tr>
<td>Dehydrogenase, µg TPF (10-20 cm depth)</td>
<td>4.57 (0.49)</td>
<td>4.93</td>
<td></td>
</tr>
<tr>
<td>Dehydrogenase, µg TPF (20-30 cm depth)</td>
<td>3.35 (0.43)</td>
<td>3.44</td>
<td></td>
</tr>
<tr>
<td>Total N, mg</td>
<td>1.22 (0.03)</td>
<td>1.24</td>
<td></td>
</tr>
<tr>
<td>Organic C, mg</td>
<td>7.68 (0.42)</td>
<td>7.80</td>
<td></td>
</tr>
<tr>
<td>Olsen P, µg</td>
<td>8.43 (1.41)</td>
<td>8.73</td>
<td></td>
</tr>
<tr>
<td>Olsen K, µg</td>
<td>168 (19.0)</td>
<td>158</td>
<td></td>
</tr>
<tr>
<td>Zinc, µg</td>
<td>1.39 (0.21)</td>
<td>1.35</td>
<td></td>
</tr>
<tr>
<td>Iron, µg</td>
<td>10.2 (0.70)</td>
<td>9.77</td>
<td></td>
</tr>
<tr>
<td>Copper, µg</td>
<td>1.49 (0.44)</td>
<td>1.24</td>
<td></td>
</tr>
<tr>
<td>Manganese, µg</td>
<td>9.82 (0.69)</td>
<td>9.70</td>
<td></td>
</tr>
<tr>
<td>Phenol Oxidase, µg Dopachrome</td>
<td>1.36 (0.06)</td>
<td>1.35</td>
<td></td>
</tr>
<tr>
<td>Microbial respiration, µg</td>
<td>3.34 (0.31)</td>
<td>3.94</td>
<td></td>
</tr>
<tr>
<td>Microbial biomass, µg</td>
<td>646 (50.5)</td>
<td>649</td>
<td></td>
</tr>
<tr>
<td>Readily Mineralizable C, µg</td>
<td>24.5 (2.10)</td>
<td>24.4</td>
<td></td>
</tr>
<tr>
<td>Microbial biomass C / Organic C</td>
<td>852 (60.1)</td>
<td>848</td>
<td></td>
</tr>
<tr>
<td>OC (Mic. respiration Mic. biomass⁻¹)</td>
<td>0.005 (.0008)</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Porosity, mg</td>
<td>5.07 (0.10)</td>
<td>5.04</td>
<td></td>
</tr>
</tbody>
</table>

** Soil property **

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C:N ratio</td>
<td>6.31 (0.38)</td>
<td>6.32</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>8.41 (0.03)</td>
<td>8.42</td>
<td></td>
</tr>
<tr>
<td>EC, dS m⁻¹</td>
<td>0.24 (0.03) *</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>Bulk Density, g cm⁻³</td>
<td>1.31 (0.03)</td>
<td>1.31</td>
<td></td>
</tr>
</tbody>
</table>

* Significant at p < 0.05
** Significant at p < 0.01
Dehydrogenase enzyme activity (DHEA) may indicate overall soil microbial activity (Wolinska and Stepniewsk, 2012), and represents a biological indicator of soil health (Chellemi and Porter, 2001). DHEA is expected to increase at sites where mineralization is occurring, as dehydrogenase catalyzes soil oxidation and reduction reactions carried out by soil saprophytic organisms. For 2011, DHEA was greater ($p = 0.05$) in sainfoin (8.42 µg g$^{-1}$) than in alfalfa (7.71 µg g$^{-1}$; SE=0.38) plots. For the 2012 season, comparisons of the interaction between legume species and soil depth reveal similar differences ($p = 0.006$), with greater DHEA in sainfoin (10.7 µg g$^{-1}$) than in alfalfa (9.05 µg g$^{-1}$) plots at 0-10 cm depth (SE = 0.50), suggesting greater microbial activity in the sainfoin plots. Tannins are known to negatively (Joanisse et al., 2007) or positively (B. Adamczyk et al., 2009) influence soil enzyme activity, depending on the enzyme of interest, but the specific role that CT have on soil DHEA is not well known, although our results suggest CT had a positive influence on the DHEA.

Smolander et al. (2012) examine various research investigating the influence that CT may have on C and N mineralization and soil microbial communities, most of which

---

**Table 4-2**
Pre-grazed plant biomass data from (Maughan et al., 2014).

<table>
<thead>
<tr>
<th>Pregrazing Biomass t ha$^{-1}$</th>
<th>4-Jun</th>
<th>9-Jul</th>
<th>23-Jul</th>
<th>22-Aug</th>
<th>30-Aug</th>
<th>13-Sep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa Legume</td>
<td>43.5 ± 0.3</td>
<td>63.2 ± 2.6</td>
<td>47.1 ± 4.2</td>
<td>42.6 ± 3.8</td>
<td>45.7 ± 3.3</td>
<td>41.9 ± 1.2</td>
</tr>
<tr>
<td>Mix</td>
<td>32.8 ± 2.8</td>
<td>34.1 ± 2.1</td>
<td>23.0 ± 3.1</td>
<td>32.4 ± 0.5</td>
<td>30.4 ± 3.4</td>
<td>30.6 ± 4.1</td>
</tr>
<tr>
<td>Fescue</td>
<td>36.9 ± 4.4</td>
<td>29.8 ± 4.2</td>
<td>25.3 ± 2.8</td>
<td>33.9 ± 2.0</td>
<td>32.6 ± 3.3</td>
<td>33.0 ± 2.5</td>
</tr>
<tr>
<td>Sainfoin Legume</td>
<td>59.3 ± 9.3</td>
<td>107.3 ± 1.1</td>
<td>39.8 ± 4.3</td>
<td>63.9 ± 1.5</td>
<td>35.5 ± 6.7</td>
<td>27.1 ± 5.4</td>
</tr>
<tr>
<td>Mix</td>
<td>31.5 ± 1.9</td>
<td>34.3 ± 5.0</td>
<td>22.7 ± 2.0</td>
<td>30.3 ± 1.8</td>
<td>27.1 ± 1.7</td>
<td>28.6 ± 1.3</td>
</tr>
<tr>
<td>Fescue</td>
<td>36.7 ± 3.5</td>
<td>32.8 ± 3.6</td>
<td>22.1 ± 1.3</td>
<td>29.4 ± 0.5</td>
<td>28.5 ± 3.2</td>
<td>25.5 ± 1.8</td>
</tr>
</tbody>
</table>
indicate inhibitory effects. Yet, our data revealed no significant differences in microbial responses between alfalfa and sainfoin plots. None of our other measured soil parameters revealed significant differences regarding greater mineralization or immobilization between the two legume species (Table 4-1), nor were there any other interaction effects.

3.1.2. Planting Configuration Effects on Soil

We hypothesized that legume monoculture (Mono) plots would decrease- while grass Mono plots would increase immobilization. Consistent with our hypothesis, differences between planting configuration suggests greater mineralization in legume than in TF monocultures with 4.2-fold greater NO$_3^-$ concentration ($p = 0.053$) at 0-30 cm at the end of the 2012 season. At 60-90 cm depth, legume Mono tended ($p = 0.07$) to present 2.5-fold greater concentrations of NO$_3^-$ than grass-legume mixtures (Mix) while NO$_3^-$ was present in TF Mono at trace amounts only. These results support other studies showing greater N-availability in leguminous systems (Cadisch et al., 1994), yet contradict results discussed by Hooper and Vitousek (1998) where they expected greater N immobilization in perennial bunchgrasses and observed varying results, in some cases N-fixing legumes showed greater immobilization than bunchgrasses. Our data also showed legumes having greater ($p = 0.03$) soil P, further supporting greater mineralization. Legume Mono showed greater ($p = 0.03$) microbial efficiency ($q$CO$_2$) than TF Mono, indicating greater substrate quality in legume treatments which is also supported by lower ($p = 0.002$) C:N ratios. The lack of difference in $q$CO$_2$ between legumes (section 3.1.1) suggests that CT were not affecting the ability of microbes to process organic matter. Legume Mono showed greater ($p = 0.03$) phenol oxidase activity
than TF Mono, indicating the presence of phenolic molecules (Sinsabaugh, 2010), yet there were no differences in microbial activity between the two legumes (section 3.1.1.).

Interestingly, TF Mono had greater \( p = 0.005 \) microbial respiration and DHEA \( p = 0.05 \) than legume Mono, indicating possible soil microbial inhibition by both CT and saponins, consistent with other studies showing terpenes having similar effects as CT on soil nutrient cycling (S. Adamczyk et al., 2013). Alternatively, lower microbial respiration and DHEA in legume plots could indicate C-limitation. In plant-biodiverse experimental plots Eisenhauer et al. (2010) found overall greater microbial respiration and microbial biomass in plots containing grasses than plots containing legumes, while the plots with legumes had greater microbial efficiency. Zak et al. (2003) evaluate soil microbial activity with varying plant species and found greater microbial respiration with increased plant diversity, which was largely due to increased plant production. Their experiment included *Lespedeza capitata*, known to contain CT, among other legumes, grasses, forbs, and trees, yet the study did not distinguish between individual species or the inherent PSM and the ensuing effects on soil microbial activities. To our knowledge there is no research regarding PSM in pasture agroecosystems with grasses and legumes.

Readily mineralized C was greater \( p = 0.002 \) in TF Mono treatments, and organic C was greater \( p = 0.04 \) in grass/legume mixtures, further supporting our hypothesis of greater C storage and N immobilization with the incorporation of grasses. As legumes are known to use more soil K, unsurprisingly TF Mono had greater K \( p = 0.005 \) than both legume Mono and Mix treatments. Other measured soil parameters showed no significant differences between planting configurations (Table 4-1), and there were no interaction effects.
3.1.3. Plant Responses

To determine whether pasture forages produce enough PSM to influence soil nutrient cycling we measured saponins from alfalfa and CT from sainfoin. Prior to grazing, baseline data from plant analyses in May, 2010 showed a 0.93-fold greater (6.32 g kg⁻¹) concentration of saponins in alfalfa/grass Mix treatments than in Mono (3.27 g kg⁻¹; \( p = 0.03; \) SE = 0.06), indicating greater C resources in alfalfa/grass plots, supporting soil organic C data from Section 3.1.2. Similarly, saponin concentration in alfalfa during the 2012 season were 0.46-fold greater in the Mix than in Mono treatments (\( p = 0.06; \) Table 4-3), which contradicts Clemensen et al. (2018) where there were no differences in saponin concentration when alfalfa grew in monocultures or two-way mixtures. Saponins tended (\( p = 0.07 \)) to be greater in June than in Aug. and Sept. There were no differences in CT concentration in sainfoin between planting configurations across the 2 years of the study, yet CT concentration was greater (\( p = 0.02 \)) in June and Aug. than in Sept. Plant secondary metabolites are known to fluctuate over time (Cheeke, 1998; Tava, et al., 1999; Clemensen et al., 2017), and peak concentrations may subsequently have more influence on soil nutrient cycling. Our soil collection in 2012 was in Sept. when both saponins in alfalfa and CT in sainfoin were at their lowest concentrations, possibly weakening the potential effects of these PSM in the field. On the other hand, cumulative effects could presumably be greater, depending on the rate of turnover of the PSM in the soil.
Table 4-3
Field experiment 2012 plant chemical results between different planting configurations; monocultures (Mono) and grass/legume mixtures (Mix), and across time. Nitrogen results for TF include combined means of both legumes followed by combined means for each legume.

<table>
<thead>
<tr>
<th>Plant property</th>
<th>Planting Config. (SE)</th>
<th>Month (SE)</th>
<th>Legume (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mono</td>
<td>Mix</td>
<td>June</td>
</tr>
<tr>
<td><strong>Alfalfa</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saponins, g kg(^{-1})</td>
<td>4.63 (1.44)</td>
<td>6.78</td>
<td>7.21 (1.59)</td>
</tr>
<tr>
<td>Nitrogen, g kg(^{-1})</td>
<td>46.3 (3.96)</td>
<td>44.3</td>
<td>43.7 (3.81)</td>
</tr>
<tr>
<td><strong>Sainfoin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tannins, g kg(^{-1})</td>
<td>58.4 (2.61)</td>
<td>59.3</td>
<td>60.6 (2.88) * a</td>
</tr>
<tr>
<td>Nitrogen, g kg(^{-1})</td>
<td>40.4 (0.34)</td>
<td>39.8</td>
<td>41.0 (0.49) * a</td>
</tr>
<tr>
<td><strong>Tall Fescue</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrogen, g kg(^{-1})</td>
<td>27.9 (0.59)</td>
<td>26.0</td>
<td>28.6 (0.94)</td>
</tr>
<tr>
<td>w/Alfalfa</td>
<td>29.0 (0.84)</td>
<td>27.5</td>
<td>30.1 (1.34)</td>
</tr>
<tr>
<td>w/Sainfoin</td>
<td>26.7 (0.84)</td>
<td>24.6</td>
<td>27.1 (1.34)</td>
</tr>
</tbody>
</table>

* Significant at p < 0.05

Concentration of CT in sainfoin during the study averaged 60.0 g kg\(^{-1}\), whereas concentration of saponins in alfalfa averaged 5.4 g kg\(^{-1}\). This substantial difference in PSM concentration between alfalfa and sainfoin could help explain the considerable difference in soil NO\(_3\)\(^-\) concentration between plots of the two legumes, such that saponins were not produced in sufficient amounts to influence soil nutrient cycling. S. Adamczyk et al. (2013) found that larger terpenes (i.e. saponins) show similar patterns of decreased soil N mineralization and nitrification as seen with monoterpenes.

Differences in total N concentration for TF growing in mixtures with either alfalfa or sainfoin were non-significant in 2010, but combined means showed total N content in TF was greater (\(p = 0.03\)) when TF was growing adjacent to alfalfa than when TF was growing adjacent to sainfoin plots in 2012 (Table 4-3), indicating greater plant-available
N from alfalfa plots. Prior research conducted by Ta and Faris (1987) showed that alfalfa ‘excretes’ more N than other legumes. Nevertheless, our study revealed that total N content in TF did not differ significantly when the plant grew adjacent to alfalfa or to sainfoin for each individual month (Table 4-3), even when differences in plant biomass between sainfoin and alfalfa Mix treatments were negligible (Table 4-2). These results further support our soil NO$_3^-$ data from section 3.1.1, and lessen the probability that NO$_3^-$ differences between alfalfa and sainfoin were based solely on plant biomass differences.

Total N concentration in sainfoin fluctuated over time ($p = 0.02$) with greater N content in June and Sept. than in August (Table 4-3), but there were no differences in total N concentration for TF or alfalfa N over time, which is in contrast to Clemensen et al. (2017) findings on significant fluctuations in total N in both TF and alfalfa over time. There were no differences in N content for alfalfa or TF between planting configurations in 2010 or 2012. There also were no interaction effects in the data.

The limitations of our field experiment include the timing of our soil collection coinciding with a difference in plant biomass between the two legumes, in addition to the end of the growing season when PSM decline in concentration, which was the case in our forages.

3.1.4. Incubation Study

To determine how cattle feces from our field experiment might have influenced soil nutrient cycling, we measured NO$_3^-$, NH$_4^+$, and DHEA over 56 days (n=8), and total C and N on days 0 and 56 (n=2), of a soil-feces incubation study. We used cattle feces from animals consuming diets of saponin-containing alfalfa and CT-containing sainfoin.
from our field experiment, at low and high rates, and a control without added manure. Both feces types had relatively low C:N ratios (< 18; section 2.2), which generally results in net mineralization (i.e. Robertson and Groffman, 2007). On day 0 there were no differences in C:N ratios between treatments. We hypothesized that feces from diets containing sainfoin with CT would reduce N mineralization given the high reactivity of CT with N. However, high feces application rates from both diets decreased mineralization, with greater overall NO₃⁻ content ($p < 0.0001$) in low-rate and control treatments than in high-rate treatments, and no significant differences between feces types (Fig. 4-1), nor any interaction effects. Our results support those of other soil manure incubation studies showing initial immobilization of N (Baitilwake et al., 2012; Probert et al., 2005), yet are in contrast to results from Abbasi et al. (2007). These differences are likely explained by differences in the C:N ratio of the manure used. Manure used by Baitilwake et al. (2012) was 19 and Probert et al. (2005) studied manures (n=45) with an average C:N ratio of 21, while that used by Abbasi et al. (2007) was only 4:1.
Incubation study - nitrate results on two rates of feces (high / low) from two different diets of either alfalfa (Alf) or sainfoin (Sain) measured 8 times over 56 days. Mineralization in the high-rate feces treatments was delayed, suggesting increased immobilization from days 3-21, and towards the end of the incubation study NO$_3^-$ increased in high-rate feces treatments while NO$_3^-$ decreased in low-rate feces treatments. This may be simply be explained by less total N applied and faster initial mineralization in low-rate feces treatments. Probert et al. (2005) allude to lignin-C causing manure to be more recalcitrant than water-soluble C and N, and Delve et al. (2001) discuss how the quality of manure is greatly influenced by what animals consume, further implying how diets with CT render feces with greater N. This suggests that incorporating CT- or saponin-containing forages into a pasture systems may slow the release of plant-available N, reducing the potential loss of N in pasture agricultural systems.

Ammonium concentration on day 0 was greater in the high-rate treatments ($p = 0.007$) than for the low-rate feces treatments, with no difference between feces types (Fig.
4-2), which may simply be because the feces contained high NH$_4^+$ which rendered greater initial concentrations of NH$_4^+$ in the high-rate treatments. The NH$_4^+$ was then likely nitrified to NO$_3^-$, immobilized, fixed to exchange sites, or volatilized due to the observed increase in NO$_3^-$ concentration for all treatments from days 0 to 3. Ammonium concentration decreased for all treatments to undetectable levels from days 3 to 28, except for feces from sainfoin diets applied at high rates on day 14, then traces were observed in all treatments on days 42 and 56 (Fig. 4-2), and there were no observed interaction effects. This contradicts results shown by Baitilwake et al. (2012) and Abbasi et al. (2007) where NH$_4^+$ was detectable through most of their incubation study. The traces of NH$_4^+$ we observed on days 42 and 56 suggest NH$_4^+$ was released from exchange sites and/or that immobilized N was mineralized at this point during the incubation. Interestingly, when the sainfoin treatment at high rates showed traces of NH$_4^+$ on day 14, NO$_3^-$ concentration in this treatment was at its lowest point. Our results show decreased mineralization in high-rate feces treatments, which may suggest that feces from both CT-containing sainfoin and saponin-containing alfalfa diets inhibit mineralization thereby increasing N immobilization.
Dehydrogenase activity in our incubation study showed similar patterns to DHEA in our field experiment, with greater \( p < 0.0001 \) activity in high-rate treatments which had less \( \text{NO}_3^- \), and no differences between feces type (Fig. 4-3), with no observed interaction effects. This supports other studies showing increased DHEA with N fertilizer application (Chu et al., 2007; Cooper and Warman, 1997). Dehydrogenase activity in all treatments fluctuated significantly over time \( (p < 0.0001) \), with activity peaking on days 3 and 7. We expected both mineralization and DHEA to be reduced by CT yet in both our field experiment and incubation study DHEA increased where \( \text{NO}_3^- \) decreased. The high-rate feces application clearly incorporates more organic matter, stimulating DHEA, but there appears to be no effect from saponins or CT from feces on DHEA in this incubation study. To our knowledge there is no research evaluating effects CT or saponins might have on soil DHEA. The increased organic matter content from the high-rate feces
treatment enhancing DHEA supports observed correlations between DHEA and soil organic C (Burgos et al., 2002). We found greater ($p = 0.03$) organic C in high-rate treatments (1.20 mg g$^{-1}$), in addition to greater DHEA, than in low-rate treatments (1.06 mg g$^{-1}$; SE = 0.04).

Fig. 4-3. Incubation study – dehydrogenase results on two rates of feces (high / low) from two different diets of either alfalfa (Alf) or sainfoin (Sain) measured 8 times over 56 days.

Total N and organic C were measured at the beginning and end of the incubation study (days 0 and 56; Fig. 4-4). Combined means of total N concentration increased ($p = 0.0004$) from day 0 (1.05 mg g$^{-1}$) to day 56 (4.36 mg g$^{-1}$; SE = 0.80), which is consistent with other incubation studies (Abbasi et al., 2007; Baitilwake et al., 2012). The most notable difference was in the low-rate feces from the sainfoin diet which increased in total N ($p = 0.01$) from 0.84 to 7.08 mg g$^{-1}$. This suggests there is more N being retained in the sainfoin treatment applied at low rates, which was unanticipated as we expected the
high-rate treatments to retain more N. There were no differences in organic C from day 0 to 56, but total N increased in all treatments, therefore C:N ratio decreased ($p < 0.0001$) in all treatments, with combined means showing a decrease from 11.9 on day 0 to 3.94 on day 56, indicating increased mineralization in all treatments over the course of the incubation study. There were no observed interaction effects.

![Total N, Organic C, & C:N at Day 0 & 56](image)

**Fig. 4-4.** Incubation study – total nitrogen, total organic carbon, and C:N ratio on two rates of feces (high / low) from two different diets of either alfalfa or sainfoin measured at the beginning and end of the study (days 0 and 56).

Our assumptions are based in part on the presence of PSM in cattle feces. We analyzed CT and saponins in the shoot tissues of plant samples but did not include measurements of CT or saponins in feces, soil, or root tissues of plants. However, recent results from our lab suggest that CT concentration in cattle feces is proportional to the concentration of CT in sainfoin (Stewart, 2018). Further research is needed to detect specific PSM in root tissues, surrounding soil, and fecal samples in pasture agricultural
systems. Research is also needed to clarify the role that tannins and terpenes may have on DHEA specifically, as our data offered mixed results.

4. Conclusions

Plant secondary metabolites such as tannins and terpenes (i.e. saponins) reduce soil mineralization in silviculture systems. To our knowledge this is the first study to investigate whether pasture forages produce sufficient PSM to influence soil nutrient cycling in grazed agricultural systems. Data from our field and incubation experiments suggest CT from sainfoin and saponins from alfalfa may influence soil nutrient cycling by decreasing N mineralization. Although we found greater NO$_3^-$ content in alfalfa than in sainfoin plots, we did not observe differences in total N concentration between legumes. On the other hand, plant biomass was greater in alfalfa than in sainfoin plots at sampling time which perhaps at least partially explains the greater concentration of NO$_3^-$ in the alfalfa plots. With greater microbial respiration and DHEA in TF monocultures, we suspect that both CT and saponins from sainfoin and alfalfa, respectively, may inhibit soil microbial activity in pasture systems, although this could be explained by C-limitation in legumes. The lack of differences in soil microbial activity between plots of the two legumes, in addition to our incubation study which showed delayed mineralization from treatments that applied feces from animals consuming either alfalfa or sainfoin at high rates, supports our hypothesis that CT and saponins may reduce mineralization. The subsequent release of N later in the incubation study, depicted by an increase in NO$_3^-$, shows how implementing the use of forages containing CT and/or saponins in pasture agroecosystems may be advantageous at minimizing N loss.
This study explores a novel concept of management in pasture agricultural systems and offers a foundation for potential research investigating possibilities to increase soil nutrient retention in agroecosystems by implementing forages containing PSM. Nitrogen loss in pasture agricultural systems is problematic. The incorporation of tanniferous or terpene-containing forages could mitigate the loss of N by retaining N through increased immobilization. In Canada the tanniferous shrub *Kalmia angustifolia*, with tannin levels > 200 mg g⁻¹ (Joanisse et al., 2007), increased nutrient retention in soils which suppressed growth of other species, and when *Kalmia* was removed from the system the release of nutrients had “an effect fertilizer cannot emulate” (LeBel et al., 2008).

We conclude that N loss in pasture agricultural systems may be reduced by incorporating tanniferous and/or terpene-containing forages. This in turn can increase N and C sequestration potential of pasture agricultural soil and lessen the eutrophication of aquatic environments from N loss. This novel concept of increasing microbial immobilization in pasture agriculture systems by incorporating tanniferous or terpene-containing forages could apply to other agricultural systems.

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CHAPTER 5

CONCLUSION

The objectives of this dissertation were to better understand the temporal dynamics of the concentration of plant secondary metabolites (PSM) in plants and the influence of these chemicals on soil function in agroecosystems. I explored how planting configuration (monocultures vs. two-way mixtures) and different fertilizers (urea and feces) influenced PSM and total nitrogen content in forages. I observed increases in alkaloid concentrations (a nitrogen-containing PSM) when endophyte-infected tall fescue grew adjacent to legumes like alfalfa and birdsfoot trefoil, indicating greater nitrogen availability to the grass. Yet, when all forages were fertilized with urea, total nitrogen contents in the plants’ tissues increased while PSM concentrations were unaffected. This perhaps indicates that the presence of another plant species may trigger the production of PSM rather than increased nitrogen availability. I also observed greater concentration of saponins (a carbon-based PSM) in alfalfa when growing adjacent to tall fescue grass, indicating greater availability of carbon resources, yet data was inconclusive across studies. This further implies that resource availability likely has little to do with PSM production. Throughout all experiments conducted in this work, PSM appeared to fluctuate unpredictably. The only clear pattern emerging from this study regarding changes in PSM concentrations involved seasonal fluctuations, showing greater contents during June and July and declines towards Sept. and Oct., with diurnal differences in birdsfoot trefoil showing greater concentrations of condensed tannins in the morning than
in the evening. Thus, as environments are in constant flux, and land management objectives vary, I recommend site-specific studies in which to gauge PSM fluctuations.

Extensive research has investigated herbivore-induced responses in plants (see Ian Baldwin, Max Planck Institute, Jenna, Germany), which appear to have much greater influence on fluctuations in PSM concentrations than planting configuration or fertilization. When I began my research I observed established adjacent monoculture plots of alfalfa and birdsfoot trefoil where alfalfa weevil had defoliated alfalfa leaves, yet birdsfoot trefoil plants were unaffected by insect infestation. The birdsfoot trefoil variety used in that study was ‘Goldie’, which had high levels of condensed tannins. This observation was also noted by Colorado State University Extension Entomologist Whitney Cranshaw (J. MacAdam, personal communication, 2007). Thus, a passion for investigating alternative approaches to agriculture was born. Plant secondary metabolites act as pesticides in plants, and utilizing forages containing these metabolites can reduce our input of potentially harmful concoctions of chemicals into the environment.

Plant secondary metabolites are also beneficial to foraging animals in moderate doses and in different combinations. Empirical evidence shows that when foraging animals consume different PSM in strategic sequences they consume more and have better overall health. I discovered from an *in vitro* study that the alkaloid ergovaline binds with saponins from alfalfa in an aqueous solution, which could mitigate the negative effects of fescue toxicosis. These experiments included two different legumes with theoretical intentions of alleviating fescue toxicosis. Further research investigating different forage combinations to reduce fescue toxicosis is needed, in addition to research determining whether binding of ergovaline with saponins and/or with condensed tannins
actually occurs in the rumen using rumen fluid as a medium for incubation instead of a water solution. This sets the stage for further investigation of potential options regarding sequence (rotational) grazing to enhance the health of foraging animals and also to reduce the risk of ailments such as fescue toxicosis. My research showed lower ergovaline contents in endophyte-infected tall fescue when grown in monocultures than when grown in legume mixtures, implying that animals may benefit from systems which allow them to graze monoculture patches of endophyte-infected tall fescue and alfalfa or birdsfoot trefoil rather than grass-legume mixtures. Properly managed grazing circuits, which include a variety of plant species, can be beneficial to grazing animals.

Ongoing research is revealing the seemingly endless benefits of PSM in agroecosystems, both for plants and for the animals that consume them. My research also plunged into the realm of soil – hypothesizing that PSM influence chemical and biological processes occurring at this level. I found evidence suggesting condensed tannins from sainfoin and saponins from alfalfa decrease nitrogen mineralization in agroecosystems, subsequently reducing nitrogen loss and storing plant-available nitrogen for future use. This in turn can increase nitrogen and carbon sequestration potential in animal agricultural soil, while decreasing the eutrophication of aquatic environments from nitrogen leaching. Our current methods of industrialized agriculture are efficient but yet, not sustainable. As Franklin D. Roosevelt wrote in his letter to all State Governors on a Uniform Soil Conservation Law in 1937, “the Nation that destroys its soil destroys itself”. Different methods of agriculture need to be developed which focus not only on mass production but include a greater focus on improving the health of soil. Incorporating tanniferous and terpene-containing forages in agroecosystems could mitigate nitrogen
loss by retaining nitrogen through increased immobilization. This novel concept of increasing microbial immobilization in pasture agroecosystems by incorporating tanniferous and terpene-containing forages could apply to other agricultural systems.

Implications of this research involve improving the health of soils, plants, animals (including humans), and our environment by increasing the biodiversity in agroecosystems. Findings from this dissertation lay the groundwork for future studies investigating the interactive effects of different plant species and their inherent PSM contents, and exploring novel ways to manipulate our management tactics in order to benefit from a diversity of forage species and chemicals, distributed across the landscape in response to different agricultural objectives.
Collecting and preparing plant samples:

1. Collect 50-100g of fresh vegetative material.

2. Freeze-dry or oven-dry plant samples.

3. Grind samples to pass through a 1mm screen.

Extracting Saponins to create standard:

*note: extraction of saponins for the standard requires 25-50g dried ground material, depending on how much pure saponin desired (25g plant material renders roughly 0.2111g saponin).

STEP A: Soxhlet Extraction

1. Measure 50g ground alfalfa and place in paper thimble.

2. Extract with HPLC grade hexane (~1000mL) using soxhlet method for ~48 hours.
   
   Discard hexane.

3. Remove thimble and set out to dry overnight.

4. Continue soxhlet extraction using methanol (~2500mL) for ~48 hours. Keep extraction with methanol solvent, discard thimble with plant residue.

STEP B: Chloroform Extraction

1. Use a rotary evaporator (roto-vap) to remove excess methanol solvent (~1.5 hours).
2. Add 600mL of 50/50 chloroform/distilled water to extract and decant into a separation funnel.

3. Drain lower chloroform layer to discard.

*note: a lipid layer forms between the top aqueous solution and bottom chloroform layer of this extraction. KEEP this lipid layer with the top aqueous solution, as it likely contains saponins. This will be important in the small-scale extractions as well.

4. Add 300mL of chloroform, shake and let settle. Drain lower chloroform layer to discard.

5. Repeat step 4 for a total of 4 chloroform extractions.

STEP C: Butanol Extraction

1. Combine 300mL N-butanol with 100mL distilled water in separation funnel. Shake and let settle. Drain lower layer of water to discard.

2. Mix 100mL of water-saturated N-butanol from step C1 with aqueous solution from step B and 1.5g sodium chloride in separation funnel.

3. Drain lower polar layer and set aside to extract.

4. Drain remaining non-polar layer and set aside to keep.

5. Add 100mL water-saturated N-butanol with polar layer from step C3 back into separation funnel. Mix then let settle.

6. Repeat steps 3 and 4 for a total of 3 butanol extractions, each time keeping the accumulating non-polar layer.

7. Roto-vap the non-polar extract to dryness.
*note: the N-butanol evaporates relatively quickly under low heat, then the remaining extract takes much longer with increasing heat.

STEP D: Purifying Saponin Extract

*note: the more methanol used to dissolve residue, the lower the total yield of purified saponins.

1. Add ~ 4mL methanol to flask and sonicate to dissolve all dry residue.

2. Introduce drop-wise into acetone (~ 900mL).

3. Butchner funnel acetone to isolate precipitate. Add precipitate back into flask with ~ 2mL methanol, sonicate and drip into fresh acetone (~ 900mL).

4. Repeat step 3 (2X) and after the fourth and final time, allow the precipitate to stand in acetone for 16 hours.

5. Collect the precipitate by using a butchner funnel and allow it to dry overnight at room temperature. Yield = .2111g purified saponin.

STEP E: Creating Standard Curve

*Note: Any glassware, especially the test tubes and caps, used for the entire procedure must be washed and rinsed properly to remove all residual soap or it will confound results.

1. Measure 10mg saponin extract and add to 10mL distilled water in 16mL glass centrifuge test tube.

2. Using a serial dilution create a 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, etc. in 7 (exact brand as the first) test tubes.

3. Shake for 2 minutes and let settle for 15-20 minutes. Measure and record foam.
4. Using excel obtain an R² value (≥ 0.97) for the curve.

**Determining saponin % using small-scale extractions:**

Method developed by Steven Lee, Dale Gardner, and Andrea Clemensen

**STEP A: Methanol Extraction**

1. Weigh out 50mg of dried and ground plant material in triplicates and put into 10ml screw-cap test tubes.

2. Add 5mL methanol and put on roto-geni for ~30 minutes.

3. Centrifuge for 5 minutes at 2500 rpm.

4. Pipette or decant supernatant into newly labeled 10mL screw-cap test tubes.

5. Repeat steps 2-4 for a total of 2 methanol extractions.

6. Dry down 10ml methanol extractions.

**STEP B: Chloroform Extraction**

*Note: use teflon caps and glass pipettes for chloroform extractions.*

1. Add 5mL distilled water and 5mL chloroform to each sample from step A. Put on roto-geni for 30 minutes.

2. Centrifuge 5 minutes at 2500rpm.

3. Pipette lower chloroform layer out to discard.

4. Add 5ml chloroform to each vial, roto-geni for 30 minutes, then centrifuge 5 minutes at 2500rpm.
5. Repeat steps 3-4 for a total of 4 chloroform extractions. Roughly 5mL of aqueous solution will remain for step C.

STEP C: N-Butanol Extraction

1. Combine 150mL N-butanol with 50mL distilled water and 0.75g sodium chloride in separation funnel. Shake and let settle. Drain lower layer of water to discard.

2. Add 5ml water-saturated N-butanol (from step C1) to each aqueous sample from step B5.

3. Roto-geni for 30 minutes, then centrifuge 5 minutes at 2500rpm.

4. Pipette upper non-polar layer out and put into new and labeled 16mL test tubes.

*Note: these test tubes will be used for the foam test so they must be the same brand used in creating the standard curve.

5. Continue extracting lower polar layer by adding 5ml water-saturated N-butanol to each polar sample, roto-geni for 30 minutes, then centrifuge for 5 minutes at 2500rpm, repeating for a total of three N-butanol extractions.

6. Dry down each test tube containing the accumulated 15ml of non-polar N-butanol extractions.

STEP D: Foam Test

1. Add 5mL distilled water to each sample and roto-geni for 30 minutes.

2. Hand-shake each sample vigorously for 2 minutes. Let stand for 5-10 minutes then measure and record foam.
3. Calculate the percent of saponins by calculating mg/ml based on the standard curve equation (for instance, if the 0.25mg/ml from the standard rendered 2.8 cm of foam, use the number 0.089 by dividing 0.25 by 2.8), multiply by the cm of foam the sample rendered, multiply by 5, divide by the weight of the sample used in the extraction, lastly multiply by 100 for a percent by weight saponin content.

*Note: foam may be reduced (if a re-measurement is required) by centrifuging.

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- Laboratory experience isolating and purifying extractable condensed tannins from *Lotus corniculatus* and *Onobrychis viciifolia* using methods developed by Terrill et al. (1992) and Reed (1986). Analyzed small-scale tannin extractions for several student and professor colleagues.
- Laboratory experience isolating and purifying saponins from *Medicago sativa* using a modification of Lee et al. (2001) using methods developed by Patamalai et al. (1990) and Wall et al. (1952). Developed a suds-test extraction method for small-scale saponin extractions with Drs. Steven Lee and Dale Gardner at the USDA ARS Poisonous Plant Laboratory in Logan, UT.
- Laboratory experience analyzing soil enzymes; dehydrogenase, phosphatase, and phenol oxidase using methods developed by Tabatabai (1994) and Prosser et al. (2011).
– Experienced in operating heavy farm equipment and handling livestock.

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