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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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ABSTRACT

Understanding Plant Secondary Metabolites;

Above and Below Ground

by

Andrea K. Clemensen, Doctor of Philosophy

Utah State University, 2018

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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 ($p < 0.0001$).

(176 pages)

PUBLIC ABSTRACT

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 viciifolia* 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.

Andrea K. Clemensen

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I give special thanks to my man and life partner, Scott, for your unwavering love, patience, and encouragement. Thank you to my ladies, Sarah, Jess, Katie, and Mags, for lovingly keeping my heart light with laughter. And thank you to my favorite little lady, Fiona, for keeping my life perspective in order.

Andrea K. Clemensen

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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 (N_2O) 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 (CH_4) into the atmosphere. The agricultural sector is the largest contributor to N_2O and CH_4 emissions, both with higher global warming potential than CO_2 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 wide spread 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 CO_2 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.,

2009). 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 $cm^{3\,-1}$. 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.

References

Aazami, M.H., Tahmasbi, A.M., Ghaffari, M.H., Naserian, A.A., Valizadeh, R., Ghaffari, A.H., 2013. Effects of Saponins on Rumen Fermentation, Nutrients Digestibility, Performance, and Plasma Metabolites in Sheep and Goat Kids. Ann Rev and Res Bio. 3, 4, 596-607.

Adamczyk, B., Kitunen, V., Smolander, A., 2009. Polyphenol oxidase, tannase and
proteolytic activity in relation to tannin concentration in the soil organic horizon
under silver birch and Norway spruce. Soil Biol. Biochem. 41, 2085–2093.
https://doi.org/10.1016/j.soilbio.2009.07.018

Adamczyk, S., Kiikkilä, O., Kitunen, V., Smolander, A., 2013. Potential response of soil
processes to diterpenes, triterpenes and tannins: Nitrification, growth of
microorganisms and precipitation of proteins. Appl. Soil Ecol. 67, 47–52.
https://doi.org/10.1016/j.apsoil.2013.02.009

Adel, M.M., Sehnal, F., Jurzysta, M., 2000. Effects of alfalfa saponins on the moth
Spodoptera littoralis. J. Chem. Ecol. 26, 1065–1078.

Adler, J., 2011. The growing menace from superweeds. Sci. Am. 304, 74–79.

AlSaadawi, I.S., 1988. Biological suppression of nitrification by selected cultivars of
Helianthus annum L. J. Chem. Ecol. 14, 733–741.
https://doi.org/10.1007/BF01018768

Anderson, J.P.E., Domsch, K.H., 1978. A physiological method for the quantitative
measurement of microbial biomass in soils. Soil Biol. Biochem. 10, 215–221.

Anderson, T.-H., Domsch, K.H., 1989. Ratios of microbial biomass carbon to total
organic carbon in arable soils. Soil Biol. Biochem. 21, 471–479.

Anderton, N., Cockrum, P.A., Colegate, S.M., Edgard, J.A. Flower, K., 1999.
Assessment of potential for toxicity of *Phalaris* spp. via alkaloid content
determination: *P. coerulescens*, a case example. Phytochem Anal. 10, 113-118.

Arikan, O.A., Mulbry, W., Rice, C., 2009. Management of antibiotic residues from
agricultural sources: Use of composting to reduce chlortetracycline residues in

beef manure from treated animals. J. Hazard. Mater. 164, 483–489.

https://doi.org/10.1016/j.jhazmat.2008.08.019

Atsatt, P.R., O'Dowd, D.J., 1976. Plant defense guilds. Science 193, 24–29.

Augustin, J.M., Kuzina, V., Andersen, S.B., Bak, S., 2011. Molecular activities,

biosynthesis and evolution of triterpenoid saponins. Phytochemistry 72, 435–457.

https://doi.org/10.1016/j.phytochem.2011.01.015

Azevedo, M.D., Welty, R.E., 1995. A Study of the Fungal Endophyte Acremonium

coenophialum in the Roots of Tall Fescue Seedlings. Mycologia 87, 289.

https://doi.org/10.2307/3760825

Baldwin, I.T., 1988. Short-term damage-induced increases in tobacco alkaloids protect

plants. Oecologia. 75, 367-370.

Barbehenn, R.V., Jaros, A., Lee, G., Mozola, C., Weir, Q., Salminen, J.-P., 2009. Tree

resistance to Lymantria dispar caterpillars: importance and limitations of foliar

tannin composition. Oecologia 159, 777–788. https://doi.org/10.1007/s00442-

008-1268-7

Barbehenn, R.V., Jones, C.P., Hagerman, A.E., Karonen, M., Salminen, J.-P., 2006a.

Ellagitannins have Greater Oxidative Activities than Condensed Tannins and

Galloyl Glucoses at High pH: Potential Impact on Caterpillars. J. Chem. Ecol. 32,

2253–2267. https://doi.org/10.1007/s10886-006-9143-7

Barbehenn, R.V., Jones, C.P., Karonen, M., Salminen, J.-P., 2006b. Tannin Composition

Affects the Oxidative Activities of Tree Leaves. J. Chem. Ecol. 32, 2235–2251.

https://doi.org/10.1007/s10886-006-9142-8

Barry, T.N., McNabb, W.C., 1999. The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. Br. J. Nutr. 81, 263–272.

Bauer, A., Black, A.L., 1981. Soil Carbon, Nitrogen, and Bulk Density Comparisons in Two Cropland Tillage Systems after 25 Years and in Virgin Grassland1. Soil Sci. Soc. Am. J. 45. https://doi.org/10.2136/sssaj1981.03615995004500060032x

Berenbaum, M.R., 1995. The chemistry of defense: theory and practice. Proc. Natl. Acad. Sci. 92, 2–8.

Beuselinck, P.R., Sleper, D.A., Bughrara, S.S., Roberts, C.A., 1992. Effect of Mono and Mixed Culture of Tall Fescue and Birdsfoot Trefoil on Yield and Quality. Agron. J. 84. https://doi.org/10.2134/agronj1992.00021962008400020002x

Blagg, B.S.J., Jarstfer, M.B., Rogers, D.H., Poulter, C.D., 2002. Recombinant Squalene Synthase. A Mechanism for the Rearrangement of Presqualene Diphosphate to Squalene. J. Am. Chem. Soc. 124, 8846–8853. https://doi.org/10.1021/ja020411a

Blake, G.R., 1965. Bulk Density1, in: Methods of Soil Analysis. Part 1. Physical and Mineralogical Properties, Including Statistics of Measurement and Sampling, Agronomy Monograph. American Society of Agronomy, Soil Science Society of America, Madison, WI, pp. 374–390.

Bradley, R.L., Titus, B.D., Preston, C.P., 2000. Changes to mineral N cycling and microbial communities in black spruce humus after additions of $(NH_4)_2SO_4$ and condensed tannins extracted from *Kalmia angustifolia* and balsam fir. Soil Biol. Biochem. 32, 1227–1240.

Brady, N.C. and Weil, R.R. 2002. The Nature and Properties of Soils. 13th ed. Prentice Hall Publishing. USA.

Bryant, J.P., Chapin, F.S., Klein, D.R., 1983. Carbon/Nutrient Balance of Boreal Plants in Relation to Vertebrate Herbivory. Oikos 40, 357. https://doi.org/10.2307/3544308

Bryant, J.P., Tuomi, J., Niemala, P., 1988. Chapter 12 - Environmental Constraint Of Constitutive and Long-Term Inducible Defenses in Woody Plants. in: Spencer, K.C., (Ed) Chemical Mediation of Coevolution. Academic Press, pp. 367–389.

Burritt, E.A., Provenza, F.D., 2000. Role of toxins in intake of varied diets by sheep. J. Chem. Ecol. 26, 1991–2005.

Catanese, F., Distel, R.A., Villalba, J.J., 2014. Effects of supplementing endophyte-infected tall fescue with sainfoin and polyethylene glycol on the physiology and ingestive behavior of sheep. J. Anim. Sci. 92, 744–757. https://doi.org/10.2527/jas.2013-6713

Center for Disease Control (CDC), 2013. Antibiotic Resistance Threats in the United States. http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf. pp. 36-37 (accessed April 2014).

Charlton, A.J., Davis, A.L., Jones, D.P., Lewis, J.R., Davies, A.P., Haslam, E., Williamson, M.P., 2000. The self-association of the black tea polyphenol theaflavin and its complexation with caffeine. J. Chem. Soc. Perkin Trans. 2 317–322.

Cheeke, P.R., 1998. Natural toxicants in feeds, forages, and poisonous plants. Danville, Ill. : Interstate Publishers.

Chellemi, D.O., Porter, I.J., 2001. The role of plant pathology in understanding soil health and its application to production agriculture. Australas. Plant Pathol. 30, 103–109.

Chichester, F.W., Chaison, R.F.J., 1992. Analysis of Carbon. In: Calcareous Soils Using A Two Temperature Dry Combustion Infrared Instrumental Procedure. Soil Sci. 153.

Christensen, M.J., Bennett, R.J., Schmid, J., 2002. Growth of Epichloë/Neotyphodium and p-endophytes in leaves of Lolium and Festuca grasses. Mycol. Res. 106, 93–106.

Clark, M.S., Horwath, W.R., Shennan, C., Scow, K.M., 1998. Changes in soil chemical properties resulting from organic and low-input farming practices. Agron. J. 90, 662–671.

Clemensen, A.K., Provenza, F.D., Lee, S.T., Gardner, D.R., Rottinghaus, G.E., Villalba, J.J., 2017. Plant Secondary Metabolites in Alfalfa, Birdsfoot Trefoil, Reed Canarygrass, and Tall Fescue Unaffected by Two Different Nitrogen Sources. Crop Sci. 57, 964-970. https://doi.org/10.2135/cropsci2016.08.0680

Coley, P.D., Bryant, J.P., Chapin, F.S., 1985. Resource Availability and Plant Antiherbivore Defense. Science 230, 895–899. https://doi.org/10.1126/science.230.4728.895

Crozier, A., Clifford, M.N., Ashihara, H., Ashihara, H., 2001. Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet. John Wiley & Sons, Incorporated, Chichester, United Kingdom.

Culvenor, C.C.J., Dal Bon, R., Smith, L.W., 1964. The occurrence of indolealkylamine alkaloids in Phalaris tuberosa L. and P. arundinacea L. Aust. J. Chem. 17, 1301–1304.

Czochanska, Z., Foo, L.Y., Newman, R.H., Porter, L.J., 1980. Polymeric proanthocyanidins. Stereochemistry, structural units, and molecular weight. J Chem Soc Perkin Trans 1 2278–2286. https://doi.org/10.1039/P19800002278

D'Addabbo, T., Carbonara, T., Leonetti, P., Radicci, V., Tava, A., Avato, P., 2011. Control of plant parasitic nematodes with active saponins and biomass from Medicago sativa. Phytochem. Rev. 10, 503–519. https://doi.org/10.1007/s11101-010-9180-2

Danso, S.K.A., Curbelo, S., labandera, C., pastorini, D., 1991. Herbage yield and nitrogen-fixation in a triple-species mixed sward of white clover, lotus and fescue. Soil Biol. Biochem. 23, 65–70. https://doi.org/10.1016/0038-0717(91)90163-E

Das, R., Steege, A., Baron, S., Beckman, J., Harrison, R., 2001. Pesticide-related illness among migrant farm workers in the United States. Int J of Occup and Environ Health 7, 4, 303–312.

Davidson, E.A., Galloway, L.F., Strand, M.K., 1987. Assessing available carbon: Comparison of techniques across selected forest soils. Commun. Soil Sci. Plant Anal. 18, 45–64. https://doi.org/10.1080/00103628709367802

De Geyter, E., Swevers, L., Caccia, S., Geelen, D., Smagghe, G., 2012. Saponins show high entomotoxicity by cell membrane permeation in Lepidoptera. Pest Manag. Sci. 68, 1199–1205. https://doi.org/10.1002/ps.3284

Doran, J.W., 1994. Defining soil quality for a sustainable environment: proceedings of a symposium sponsored by Divisions S-3, S-6, and S-2 of the Soil Science Society of America, Division A-5 of the American Society of Agronomy, and the North Central Region Committee on Soil Organic Matter (NCR-59) in Minneapolis, MN, 4-5 November 1992, Soil Science Society of America (Eds.), SSSA special publication. SSSA : American Society of Agronomy, Madison, Wis.

Doran, J.W., Parkin, T.B., 1996. Quantitative indicators of soil quality: a minimum data set [electronic resource]. In: Methods for Assessing Soil Quality / Editors, John W. Doran and Alice J. Jones ; Editorial Committee, Richard P. Dick ... [et Al.] ; Editor-in-Chief SSSA, Jerry M. Bigham ; Managing Editor, David M. Kral ; Associate Editor, Marian K. Viney. Madison, Wis., USA : Soil Science Society of America, 1996, pp. 25–37.

Doran, J.W., Safley, M., 1997. Defining and assessing soil health and sustainable productivity. In: Biological Indicators of Soil Health. Wallingford ; New York : CAB International, c1997, pp. 1–28.

Drinkwater, L.E., Wagoner, P., Sarrantonio, M., 1998. Legume-based cropping systems have reduced carbon and nitrogen losses. Nature 396, 262–265.

Engel, C., 2002. Wild Health: How Animals Keep Themselves Well and what We Can Learn from Them. Weidenfeld & Nicolson.

Eskenazi, B., Marks, A.R., Bradman, A., Harley, K., Barr, D.B., Johnson, C., Morga, N., Jewell, N.P., 2007. Organophosphate Pesticide Exposure and Neurodevelopment in Young Mexican-American Children. Environ. Health Perspect. 115, 792–798. https://doi.org/10.1289/ehp.9828

Facchini, P.J., 2001. Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications. Annu. Rev. Plant Biol. 52, 29–66.

Feeny, P., 1976. Plant Apparency and Chemical Defense, in: Wallace, J.W., Mansell, R.L. (Eds.), Biochemical Interaction Between Plants and Insects. Springer US, Boston, MA, pp. 1–40.

Fernández, P.L., Álvarez, C.R., Schindler, V., Taboada, M.A., 2010. Changes in topsoil bulk density after grazing crop residues under no-till farming. Geoderma 159, 24–30. https://doi.org/10.1016/j.geoderma.2010.06.010

Fleetwood, D.J., Scott, B., Lane, G.A., Tanaka, A., Johnson, R.D., 2007. A Complex Ergovaline Gene Cluster in Epichloe Endophytes of Grasses. Appl. Environ. Microbiol. 73, 2571–2579. https://doi.org/10.1128/AEM.00257-07

Foley, W.J., Iason, G.R., McArthur, C., 1999. Role of plant secondary metabolites in the nutritional ecology of mammalian herbivores: How far have we come in 25 years? In: Jung, H.G., Fahey, G.C. Jr (eds.) Nutritional Ecology of Herbivores. Proc. Vth Int. Symp. Nutr. Herb. Am. Soc. Anim. Sci., Chicago, IL. p. 130–209

Food and Agriculture Organization (FOA), 1998. Biodiversity of food and agriculture. http://www.fao.org/fileadmin/templates/biodiversity_paia/PAR-FAO-book_lr.pdf accessed (April 2013).

Food and Agriculture Organization (FAO), 2011. The International Treaty on Plant Genetic Resources for Food and Agriculture http://www.planttreaty.org/sites/default/files/edm1_full_en.pdf (accessed April 2013).

Food and Drug Administration (FDA), 2011. Summary Report on Antimicrobials Sold or

    Distributed for Use in Food-Producing Animals. Available at:

    http://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActAD

    UFA/UCM338170.pdf (accessed April 2014).

Franzluebbers, A.J., Hill, N.S., 2005. Soil carbon, nitrogen, and ergot alkaloids with

    short-and long-term exposure to endophyte-infected and endophyte-free tall

    fescue. Soil Sci. Soc. Am. J. 69, 404–412.

Freeland, W.J., Janzen, D.H., 1974. Strategies in herbivory by mammals: the role of plant

    secondary compounds. Am. Nat. 269–289.

Gavlak, R., Horneck, D., Miller, R.O., Kotuby-Amacher, J., 2003. Soil, plant and water

    reference methods for the western region. WCC-103 Publ. WREP-125 17–36.

Gershenzon, J. 1994. The cost of plant chemical defense against herbivory: a biochemical

    perspective. In: Bernays, E.A. (ed.), Insect-plant interactions. CRC Press, pp. 105-

    173.

Gilliom, R.J., 2006. Pesticides in the nation's streams and ground water, 1992--2001 : the

    quality of our nation's waters / by Robert J. Gilliom ... [et al.]. In: Circular 1291.

    Reston, VA : U.S. Geological Survey, c2006.

Golluscio, R.A., Austin, A.T., García Martínez, G.C., Gonzalez-Polo, M., Sala, O.E.,

    Jackson, R.B., 2009. Sheep Grazing Decreases Organic Carbon and Nitrogen

    Pools in the Patagonian Steppe: Combination of Direct and Indirect Effects.

    Ecosystems 12, 686–697. https://doi.org/10.1007/s10021-009-9252-6

Hamilton, J.G., Zangerl, A.R., DeLucia, E.H., Berenbaum, M.R., 2001. The carbon–

    nutrient balance hypothesis: its rise and fall. Ecol. Lett. 4, 86–95.

Harwood, R.R., 1994. Managing the living soil for human well-being. *In* Environment and Agriculture: Rethinking Development Issues for the 21st Century, S.A. Breth, ed. Morrilton, Ark.: Winrock International Institute for Agricultural Development.

Haukioja, E., Ossipov, V., Koricheva, J., Honkanen, T., Larsson, S., Lempa, K., 1998. Biosynthetic origin of carbon-based secondary compounds: cause of variable responses of woody plants to fertilization? Chemoecology 8, 133–139.

Heap, I.M., 1997. International survey of herbicide-resistant weeds. West. Soc. Weed Sci. Proc. West. Soc. Weed Sci. 50, 27–29.

Herms, D.A., Mattson, W.J., 1992. The dilemma of plants: To grow or defend [electronic resource]. Q. Rev. Biol. 67, 283–335.

Heuer, H., Schmitt, H., Smalla, K., 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. Curr. Opin. Microbiol. 14, 236–243. https://doi.org/10.1016/j.mib.2011.04.009

Hill, N.S., Schultz, L.M., Agee, C.S., Rottinghaus, G.E., 1993. Simplified sample preparation for HPLC analysis of ergovaline in tall fescue. Crop Sci. 33, 332–333.

Hill, N.S., Stringer, W.C., Belesky, D.P., 1991. Competitiveness of tall fescue as influenced by Acremonium coenophialum. Crop Sci. 31, 185–190.

Honeycutt, C.W., Griffin, T.S., Wienhold, B.J., Eghball, B., Albrecht, S.L., Powell, J.M., Woodbury, B.L., Sistani, K.R., Hubbard, R.K., Torbert, H.A., 2005. Protocols for Nationally Coordinated Laboratory and Field Research on Manure Nitrogen Mineralization. Commun. Soil Sci. Plant Anal. 36, 2807–2822. https://doi.org/10.1080/00103620500304184

Hooper, D.U., 1997. The Effects of Plant Composition and Diversity on Ecosystem
    Processes. Science 277, 1302–1305.
    https://doi.org/10.1126/science.277.5330.1302

Hooper, D.U., Vitousek, P.M., 1998. Effects of Plant Composition and Diversity on
    Nutrient Cycling. Ecol. Monogr. 68, 121. https://doi.org/10.2307/2657146

Horwath, W., 2007. 12 - Carbon Cycling and Formation of Soil Organic Matter. In: Paul,
    E.A., Soil Microbiology, Ecology and Biochemistry (Third Edition). Academic
    Press, San Diego, pp. 303–339.

Humphries, S.S., Stewart, A.J., Gwinn, K.D., 2001. Effects of endophyte status of tall
    fescue tissues on the earthworm (Eisenia fetida). Environ. Toxicol. Chem. 20,
    1346–1350.

Jayanegara, A., Togtokhbayar, N., Makkar, H.P.S., Becker, K., 2009. Tannins determined
    by various methods as predictors of methane production reduction potential of
    plants by an in vitro rumen fermentation system. Anim. Feed Sci. Technol. 150,
    230–237. https://doi.org/10.1016/j.anifeedsci.2008.10.011

Joanisse, G.D., Bradley, R.L., Preston, C.M., Munson, A.D., 2007. Soil enzyme
    inhibition by condensed litter tannins may drive ecosystem structure and
    processes: the case of Kalmia angustifolia. New Phytol. 175, 535–546.
    https://doi.org/10.1111/j.1469-8137.2007.02113.x

Johnston, A.E., Poulton, P.R., Coleman, K., 2009. Chapter 1 Soil Organic Matter, in:
    Advances in Agronomy. Elsevier, pp. 1–57.

Jones, C.G., Firn, R.D., 1991. On the evolution of plant secondary chemical diversity.
    Philos. Trans. R. Soc. Lond. B 333, 273–280.

Jones, C.G., Hartley, S.E., 1999. A Protein Competition Model of Phenolic Allocation. Oikos 86, 27. https://doi.org/10.2307/3546567

Ju, H.-J., Hill, N.S., Abbott, T., Ingram, K.T., 2006. Temperature Influences on Endophyte Growth in Tall Fescue. Crop Sci. 46, 404–412. https://doi.org/10.2135/cropsci2005.0282

Jurzysta, M., Oleszek, W., 1987. The allelopathic potential of alfalfa root medicagenic acid glycosides and their fate in soil environments. Plant Soil 98, 67.

Kallenbach, R.L., Bishop-Hurley, G.J., Massie, M.D., Rottinghaus, G.E., West, C.P., 2003. Herbage mass, nutritive value, and ergovaline concentration of stockpiled tall fescue. Crop Sci. 43, 1001–1005.

Kandeler, E., 2007. 3 - Physiological And Biochemical Methods For Studying Soil Biota And Their Function A2 - Paul, Eldor A., in: Soil Microbiology, Ecology and Biochemistry (Third Edition). Academic Press, San Diego, pp. 53–83.

Karban, R., Baldwin, I.T., 1997. Induced Responses to Herbivory. The University of Chicago Press, Chicago and London.

Kelleher, B.P., Simpson, M.J., Simpson, A.J., 2006. Assessing the fate and transformation of plant residues in the terrestrial environment using HR-MAS NMR spectroscopy. Geochim. Cosmochim. Acta 70, 4080–4094. https://doi.org/10.1016/j.gca.2006.06.012

Kemper, N., Färber, H., Skutlarek, D., Krieter, J., 2008. Analysis of antibiotic residues in liquid manure and leachate of dairy farms in Northern Germany. Agric. Water Manag. 95, 1288–1292. https://doi.org/10.1016/j.agwat.2008.05.008

Kennedy, A.C., Papendick, R.I., 1995. Microbial characteristics of soil quality. J. Soil Water Conserv. 50, 243–248.

Koricheva, J., 2002. The carbon-nutrient balance hypothesis is dead; long live the carbon-nutrient balance hypothesis? Oikos 537–539.

Kraus, T.E.C., Zasoski, R.J., Dahlgren, R.A., 2004. Fertility and pH effects on polyphenol and condensed tannin concentrations in foliage and roots. Plant Soil 262, 95–109.

Kumar, K., Gupta, S.C., Baidoo, S.K., Chander, Y., Rosen, C.J., 2005. Antibiotic Uptake by Plants from Soil Fertilized with Animal Manure. J. Environ. Qual. 34, 2082. https://doi.org/10.2134/jeq2005.0026

Lee, S.T., Vogel, K.P., Gardner, D.R., Stegelmeier, B.L., 2001. The isolation and identification of steroidal sapogenins in switchgrass [electronic resource]. J. Nat. Toxins 10, 273–281.

Lu, C.D., Jorgensen, N.A., 1987. Alfalfa Saponins Affect Site and Extent of Nutrient Digestion in Ruminants. J. Nutr. 117, 5, 919-927.

Liang, P.H., Ko, T.P., Wang, A.H. J., 2002. Structure, mechanism and function of prenyltransferases: Structure, mechanism and function of prenyltransferases. Eur. J. Biochem. 269, 3339–3354. https://doi.org/10.1046/j.1432-1033.2002.03014.x

Liebman, M., Helmers, M.J., Schulte, L.A., Chase, C.A., 2013. Using biodiversity to link agricultural productivity with environmental quality: Results from three field experiments in Iowa. Renew. Agric. Food Syst. 28, 115–128. https://doi.org/10.1017/S1742170512000300

Lorenz, K., Preston, C.M., 2002. Characterization of high-tannin fractions from humus by carbon-13 cross-polarization and magic-angle spinning nuclear magnetic resonance. J. Environ. Qual. 31, 431–436.

Lorenz, K., Preston, C.M., Raspe, S., Morrison, I.K., Feger, K.H., 2000. Litter decomposition and humus characteristics in Canadian and German spruce ecosystems: information from tannin analysis and< sup> 13</sup> C CPMAS NMR. Soil Biol. Biochem. 32, 779–792.

Luijendijk, T.J.C., van der Meijden, E., Verpoorte, R., 1996. Involvement of strictosidine as a defensive chemical in Catharanthus roseus. J. Chem. Ecol. 22, 1355–1366. https://doi.org/10.1007/BF02027718

Lyman, T.D., Provenza, F.D., Villalba, J.J., Wiedmeier, R.D., 2012. Phytochemical complementarities among endophyte-infected tall fescue, reed canarygrass, birdsfoot trefoil and alfalfa affect cattle foraging. Animal 6, 676–682. https://doi.org/10.1017/S1751731111001996

Majak, W., McDiarmid, R.E., Powell, T.W., Van Ryswyk, A.L., Stout, D.G., Williams, R.J., Tucker, R.E., 1979. Relationships between alkaloids in reed canarygrass (Phalaris arundinacea), soil moisture and nitrogen fertility. Plant Cell Environ. 2, 335–340.

Mallarino, A.P., Perdomo, C.H., West, C.P., Wedin, W.F., Goyenola, R.S., 1990. Legume species and proportion effects on symbiotic dinitrogen fixation in legume-grass mixtures. Agron. J. 82, 785–789.

Mantz, G.K., Villalba, J.J., Provenza, F.D., 2008. Supplemental polyethylene glycol affects intake of and preference for Sericea lespedeza by cattle. J. Anim. Sci. 87, 761–769. https://doi.org/10.2527/jas.2007-0706

Marten, G.C., 1973. Alkaloids in Reed Canarygrass, in: Anti-Quality Components of Forages, CSSA Special Publication. Crop Science Society of America, Madison, WI.

Marten, G.C., Hovin, A.W., Jordan, R.M., 1976. Biological significance of reed canarygrass alkaloids and associated palatability variation to grazing sheep and cattle. Agron. J. 909–914.

Marten, G.C., Larson, W.E., Clapp, C.E., 1979. Effects of municipal wastewater effluent and cutting management on persistence and yield of eight perennial forages. Am. Soc. Agron. Agron. J. 71, 650–658.

McBee, R.H., 1971. Significance of intestinal microflora in herbivory. Annu. Rev. Ecol. Syst. 165–176.

McKey, D., 1974. Adaptive patterns in alkaloid physiology. Am. Nat. 305–320.

National Research Council, National Research Council (Eds.), 2010. Toward sustainable agricultural systems in the 21st century. National Academies Press, Washington, DC.

Nennich, T.D., Harrison, J.H., VanWieringen, L.M., Meyer, D., Heinrichs, A.J., Weiss, W.P., St-Pierre, N.R., Kincaid, R.L., Davidson, D.L., Block, E., 2005. Prediction of manure and nutrient excretion from dairy cattle. J. Dairy Sci. 88, 3721–3733.

Nixon, S.W., 1995. Coastal marine eutrophication: A definition, social causes, and future concerns. Ophelia 41, 199–219. https://doi.org/10.1080/00785236.1995.10422044

Northup, R.R., Dahlgren, R.A., Yu, Z., 1995. Intraspecific variation of conifer phenolic concentration on a marine terrace soil acidity gradient; a new interpretation. Plant Soil 171, 255–262. https://doi.org/10.1007/BF00010279

Okuda, T., Mori, K., Shiota, M., 1982. Effects of interaction of tannins and coexisting substances. III Formation and solubilization of precipitates with alkaloids. Yakugaku Zasshi. 102, 9, 854-858.

Orwin, K.H., Bertram, J.E., Clough, T.J., Condron, L.M., Sherlock, R.R., O'Callaghan, M., Ray, J., Baird, D.B., 2010. Impact of bovine urine deposition on soil microbial activity, biomass, and community structure. Appl. Soil Ecol. 44, 89–100. https://doi.org/10.1016/j.apsoil.2009.10.004

Patamalai, B., Hill, D.W., Camp, B.J., Hejtmancik, E., Bridges, C.H., 1990. The isolation and identification of steroidal sapogenins in Kleingrass. Vet. Hum. Toxicol. 32, 314–318.

Pearce, N., McLean, D., 2005. Agricultural exposures and non-Hodgkin's lymphoma. Scand. J. Work. Environ. Health 18–25.

Porter, J.K., 1995. Analysis of endophyte toxins: Fescue and other grasses toxic to livestock. J. Anim. Sci. 73, 871-880.

Powers, J.C., Ponticello, I., 1968. A total synthesis of the diazaphenanthrene alkaloid perlolidine. J. Am. Chem. Soc. 90, 7102-7106.

Price, P.W., 1991. Darwinian methodology and the theory of insect herbivore population dynamics. Ann. Entomol. Soc. Am. 84, 465–473.

Prosser, J.A., Speir, T.W., Stott, D.E., 2011. Soil Oxidoreductases and FDA Hydrolysis. In Methods of Soil Enzymology. R.P. Dick (editor) Soil Science Society of America Book series No. 9, Madison, USA.

Provenza, F.D., Villalba, J.J., Haskell, J., MacAdam, J.W., Griggs, T.C., Wiedmeier, R.D., 2007. The Value to Herbivores of Plant Physical and Chemical Diversity in Time and Space. Crop Sci. 47, 382. https://doi.org/10.2135/cropsci2006.02.0083

Ramírez-Restrepo, C.A., Barry, T.N., Pomroy, W.E., López-Villalobos, N., McNabb, W.C., Kemp, P.D., 2005. Use of Lotus corniculatus containing condensed tannins to increase summer lamb growth under commercial dryland farming conditions with minimal anthelmintic drench input. Anim. Feed Sci. Technol. 122, 197–217. https://doi.org/10.1016/j.anifeedsci.2005.03.009

Reed, J.D., 1986. Relationships among Soluble Phenolics, Insoluble Proanthocyanidins and Fiber in East African Browse Species. J. Range Manag. 39, 5. https://doi.org/10.2307/3899675

Reichardt, P.B., Chapin III, F.S., Bryant, J.P., Mattes, B.R., Clausen, T.P., 1991. Carbon/nutrient balance as a predictor of plant defense in Alaskan balsam poplar: potential importance of metabolite turnover. Oecologia 88, 401–406.

Rhoades, D.F., 1979. Evolution of plant chemical defense against herbivores. In: Rosenthal, G. A., Janzen, D. H. (Eds.), Herbivores, their interaction with secondary plant metabolites, pp. 3-54. Academic Press, New York.

Rhoades, D.F., Cates, R.G., 1976. Toward a general theory of plant antiherbivore chemistry. Recent Adv. Phytochem. 168–213.

Rottinghaus, G.E., Garner, G.B., Cornell, C.N., Ellis, J.L., 1991. HPLC method for quantitating ergovaline in endophyte-infested tall fescue: seasonal variation of ergovaline levels in stems with leaf sheaths, leaf blades, and seed heads. J. Agric. Food Chem. 39, 112–115.

Salminen, J.-P., Karonen, M., 2011. Chemical ecology of tannins and other phenolics: we need a change in approach: Chemical ecology of tannins. Funct. Ecol. 25, 325–338. https://doi.org/10.1111/j.1365-2435.2010.01826.x

Salminen, J.-P., Ossipov, V., Haukioja, E., Pihlaja, K., 2001. Seasonal variation in the content of hydrolysable tannins in leaves of *Betula pubescens*. Phytochemistry 57, 15–22.

Sinsabaugh, R.L., 2010. Phenol oxidase, peroxidase and organic matter dynamics of soil. Soil Biol. Biochem. 42, 391–404. https://doi.org/10.1016/j.soilbio.2009.10.014

Smith, A.D., 1959. Adequacy of Some Important Browse Species in Overwintering of Mule Deer. J. Range Manag. 12, 8. https://doi.org/10.2307/3895208

Smith, J.L., Collins, H.P., 2007. 17 - Management of organisms and their processes in soils A2 - Paul, Eldor A., in: Soil Microbiology, Ecology and Biochemistry (Third Edition). Academic Press, San Diego, pp. 471–502.

Smith, J.L., McNeal, B.L., Cheng, H.H., 1985. Estimation of soil microbial biomass: an analysis of the respiratory response of soils. Soil Biol. Biochem. 17, 11–16.

Smolander, A., Kanerva, S., Adamczyk, B., Kitunen, V., 2012. Nitrogen transformations in boreal forest soils—does composition of plant secondary compounds give any explanations? Plant Soil 350, 1–26. https://doi.org/10.1007/s11104-011-0895-7

Stidham, W.D., Piper, E.L., Featherstone, H.E., Brown, C.J., Daniels, L.B., 1982. Toxic fescue linked to reduced milk output in ewes. Ark. Farm Res. - Ark. Agric. Exp. Stn. 31, 9–9.

Stuedemann, J.A., Hoveland, C.S., Thompson, F.N., Townsend, W.E., Ciordia, H., Belesky, D.P., Wilkinson, S.R., Breedlove, D.L., Devine, O.J., 1985. Utilization and management of endophyte-infested tall fescue: affects on steer performance and behavior. Proc. South. Pasture Forage Crop Improv. Conf. 17–20.

Subbarao, G.V., Rondon, M., Ito, O., Ishikawa, T., Rao, I.M., Nakahara, K., Lascano, C., Berry, W.L., 2007. Biological nitrification inhibition (BNI)—is it a widespread phenomenon? Plant Soil 294, 5–18. https://doi.org/10.1007/s11104-006-9159-3

Tabatabai, M.A., 1994. Soil Enzymes, in: Methods of Soil Analysis: Part 2— Microbiological and Biochemical Properties, SSSA Book Series. Soil Science Society of America, Madison, WI, pp. 775–833.

Taiz, L., Zeiger, E., 2002. Plant Physiology. Sinauer Associates, Incorporated, Publishers.

Terrill, T.H., Rowan, A.M., Douglas, G.B., Barry, T.N., 1992. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. J. Sci. Food Agric. 58, 321–329. https://doi.org/10.1002/jsfa.2740580306

Thompson, F.N., Stuedemann, J.A., Hill, N.S., 2001. Anti-Quality Factors Associated with Alkaloids in Eastern Temperate Pasture. J. Range Manag. 54, 474. https://doi.org/10.2307/4003119

Trlica, M.J., Rittenhouse, L.R., 1993. Grazing and Plant Performance. Ecol. Appl. 3, 21–23. https://doi.org/10.2307/1941783

Tuomi, J., Niemelä, P., Haukioja, E., Sirén, S., Neuvonen, S., 1984. Nutrient stress: an explanation for plant anti-herbivore responses to defoliation. Oecologia 61, 208–210.

U.S. Department of Agriculture, 2011. The Soil and Water Resources Conservation Act (RCA Appraisal). http://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/stelprdb1044939.pdf. (accessed May 2014).

U.S. Department of Agriculture, Agricultural Research Service, Natural Resources Conservation Service, Soil Quality Institute. 2001. Soil Quality Test Kit Guide. Bulk Density Test. p 9. http://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_050956.pdf

U.S. Department of Agriculture - Economic Research Service - Agricultural Trade, 2017. URL https://www.ers.usda.gov/data-products/ag-and-food-statistics-charting-the-essentials/agricultural-trade/ (accessed May 2017).

U.S. Department of Agriculture – National Resources Conservation Service, 2013. Summary Report: 2010 National Resources Inventory, Natural Resources Conservation Service, Washington, DC, and Center for Survey Statistics and Methodology, Iowa State University, Ames, Iowa. http://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/stelprdb1167354.pdf (accessed April 2014).

U.S. Environmental Protection Agency (EPA), 2017. Overview of Greenhouse Gasses. https://www.epa.gov/ghgemissions/overview-greenouse-gases (accessed March 2017).

Vasta, V., Luciano, G., 2011. The effects of dietary consumption of plants secondary compounds on small ruminants' products quality. Small Rumin. Res. 101, 150–159. https://doi.org/10.1016/j.smallrumres.2011.09.035

Vasta, V., Nudda, A., Cannas, A., Lanza, M., Priolo, A., 2008. Alternative feed resources and their effects on the quality of meat and milk from small ruminants. Anim. Feed Sci. Technol. 147, 223–246. https://doi.org/10.1016/j.anifeedsci.2007.09.020

Verpoorte, R., 1998. Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development. Drug Discov. Today 3, 232–238.

Veteli, T.O., Mattson, W.J., Niemelä, P., Julkunen-Tiitto, R., Kellomäki, S., Kuokkanen, K., Lavola, A., 2007. Do Elevated Temperature and CO2 Generally Have Counteracting Effects on Phenolic Phytochemistry of Boreal Trees? J. Chem. Ecol. 33, 287–296. https://doi.org/10.1007/s10886-006-9235-4

Villalba, J.J., Miller, J., Hall, J.O., Clemensen, A.K., Stott, R., Snyder, D., Provenza, F.D., 2013. Preference for tanniferous (Onobrychis viciifolia) and non-tanniferous (Astragalus cicer) forage plants by sheep in response to challenge infection with Haemonchus contortus. Small Rumin. Res. 112, 199–207. https://doi.org/10.1016/j.smallrumres.2012.11.033

Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H., Tilman, D.G., 1997. Technical Report: Human Alteration of

the Global Nitrogen Cycle: Sources and Consequences. Ecol. Appl. 7, 737.

https://doi.org/10.2307/2269431

Waghorn, G., 2008. Beneficial and detrimental effects of dietary condensed tannins for

sustainable sheep and goat production—Progress and challenges. Anim. Feed Sci.

Technol. 147, 116–139. https://doi.org/10.1016/j.anifeedsci.2007.09.013

Waghorn, G.C., Shelton, I.D., McNabb, W.C., McCutcheon, S.N., 1994. Effects of

condensed tannins in Lotus pedunculatus on its nutritive value for sheep. 2.

Nitrogenous aspects. J. Agric. Sci. 123, 109–119.

https://doi.org/10.1017/S0021859600067836

Wall, M.E., Eddy, C.R., McClennan, M.L., Klumpp, M.E., 1952. Detection and

estimation of steroidal sapogenins in plant tissue. Anal. Chem. 24, 1337–1341.

Wang, J., Machado, C., Panaccione, D.G., Tsai, H.-F., Schardl, C.L., 2004. The

determinant step in ergot alkaloid biosynthesis by an endophyte of perennial

ryegrass. Fungal Genet. Biol. 41, 189–198.

https://doi.org/10.1016/j.fgb.2003.10.002

Wang, K.-H., McSorley, R., Bohlen, P., Gathumbi, S.M., 2006. Cattle grazing increases

microbial biomass and alters soil nematode communities in subtropical pastures.

Soil Biol. Biochem. 38, 1956–1965. https://doi.org/10.1016/j.soilbio.2005.12.019

Wardle, D.A., Bonner, K.I., Barker, G.M., Yeates, G.W., Nicholson, K.S., Bardgett,

R.D., Watson, R.N., Ghani, A., 1999. Plant Removals in Perennial Grassland:

Vegetation Dynamics, Decomposers, Soil Biodiversity, and Ecosystem

Properties. Ecol. Monogr. 69, 535. https://doi.org/10.2307/2657230

Wedin, D.A., Tilman, D., 1990. Species effects on nitrogen cycling: a test with perennial grasses. Oecologia 84, 433–441.

Wilkes, G., Kafton, D., Yeatman, C.W., 1984. Plant genetic resources : a conservation imperative / edited by Christopher W. Yeatman, David Kafton, Garrison Wilkes., in: AAAS Selected Symposia Series ; 87. Boulder, Colo. : Published by Westview Press for the American Association for the Advancement of Science, Washington, D.C., c1984.

Wilkinson, S., 1958. 428. 5-Methoxy-N-methyltryptamine: a new indole alkaloid from Phalaris arundinacea L. J. Chem. Soc. Resumed 2079–2081. https://doi.org/10.1039/JR9580002079

Winder, R.S., Lamarche, J., Constabel, C.P., Hamelin, R.C., 2013. The effects of high-tannin leaf litter from transgenic poplars on microbial communities in microcosm soils. Front. Microbiol. 4. https://doi.org/10.3389/fmicb.2013.00290

Wink, M., Meißner, C., Witte, L., 1995. Patterns of quinolizidine alkaloids in 56 species of the genus *Lupinus*. Phytochemistry 38, 139–153.

Xie, D.-Y., Dixon, R.A., 2005. Proanthocyanidin biosynthesis – still more questions than answers? Phytochemistry 66, 2127–2144. https://doi.org/10.1016/j.phytochem.2005.01.008

Zak, D.R., Holmes, W.E., White, D.C., Peacock, A.D., Tilman, D., 2003. Plant diversity, soil microbial communities, and ecosystem function: are there any links? Ecology 84, 2042–2050.

Zakir, H.A.K.M., Subbarao, G.V., Pearse, S.J., Gopalakrishnan, S., Ito, O., Ishikawa, T., Kawano, N., Nakahara, K., Yoshihashi, T., Ono, H., Yoshida, M., 2008.

Detection, isolation and characterization of a root-exuded compound, methyl 3-(4-hydroxyphenyl) propionate, responsible for biological nitrification inhibition by sorghum (*Sorghum bicolor*). New Phytol. 180, 442–451. https://doi.org/10.1111/j.1469-8137.2008.02576.x

Zarrough, K.M., Nelson, C.J., Coutts, J.H., 1983. Relationship between tillering and forage yield of tall fescue. I. Yield. Crop Sci. 23, 333–337.

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 ergopeptine, which are metabolites

produced by the fungus. The ergopeptine 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,

Hoveland, & Radcliffe, 1992; but see Rasmussen, Parsons, Xue, & Newman, 2007).

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 (λex= 250 nm and λnm = 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.

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

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.

Compound	Sample	% Bound EV
Condensed tannins	BFT (a)	0.00
	BFT (b)	10.25
Saponins	Alf (a)	38.75
	Alf (b)	39.25

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;

Mueller-Harvey, 2006; Xie & Dixon, 2005). Collectively, the *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

Aldrich, C. G., Rhodes, M. T., Miner, J. L., Kerley, M. S., & Paterson, J. A. (1993). The effects of endophyte-infected tall fescue consumption and use of a dopamine antagonist on intake, digestibility, body temperature, and blood constituents in sheep. Journal of Animal Science, 71(1), 158–163.

Arechavaleta, M., Bacon, C. W., Plattner, R. D., Hoveland, C. S., & Radcliffe, D. E. (1992). Accumulation of ergopeptide alkaloids in symbiotic tall fescue grown under deficits of soil water and nitrogen fertilizer. Applied and Environmental Microbiology, 58(3), 857–861.

Catanese, F., Distel, R. A., & Villalba, J. J. (2014). Effects of supplementing endophyte-infected tall fescue with sainfoin and polyethylene glycol on the physiology and ingestive behavior of sheep. Journal of Animal Science, 92(2), 744–757.

Charlton, A. J., Davis, A. L., Jones, D. P., Lewis, J. R., Davies, A. P., Haslam, E., & Williamson, M. P. (2000). The self-association of the black tea polyphenol

theaflavin and its complexation with caffeine. Journal of the Chemical Society, Perkin Transactions., 2, 317–322.

Cheeke, P. R. (1998). Natural Toxicants in Feeds, Forages, and Poisonous Plants -, 2nd ed. Danville, Illinois: Interstate Publishers, Inc.

Clausen, T. P., Provenza, F. D., Burritt, E. A., Reichardt, P. B., & Bryant, J. P. (1990). Ecological implications of condensed tannin structure: A case study. Journal of Chemical Ecology, 16(8), 2381–2392.

Clemensen, A. K., Provenza, F. D., Lee, S. T., Gardner, D. R., Rottinghaus, G. E., & Villalba, J. J. (2017). Plant secondary metabolites in alfalfa, birdsfoot trefoil, reed canarygrass, and tall fescue unaffected by two different nitrogen sources. Crop Science, 57, https://doi.org/10.2135/cropsci2016.08.0680

Danso, S. K. A., Curbelo, S., Labandera, C., & Pastorini, D. (1991). Herbage yield and nitrogen-fixation in a triple species mixed sward of white clover, lotus, and fescue. Soil Biology and Biochemistry, 23, 65–70.

Flick, G. W., & Mueller, S. C. (1989). Alfalfa: quality, maturity, and mean stage of development. Department of Agronomy, College of Agriculture and Life Sciences, Cornell University Cooperative Extension, Ithaca NY. Information Bulletin 217.

Francis, G., Kerem, Z., Makkar, H. P. S., & Becker, K. (2002). The biological action of saponins in animal systems: A review. British Journal of Nutrition, 88(6), 587.

Freeland, W. J., Calcott, P. H., & Anderson, L. R. (1985). Tannins and saponin: Interaction in herbivore diets. Biochemical Systematics and Ecology, 13(2), 189–193.

Friend, M. A., Provenza, F. D., & Villalba, J. J. (2015). Preference by sheep for endophyte-infected tall fescue grown adjacent to or at a distance from alfalfa. Animal, 9(3), 516–525.

Haynes, R. J. (1980). Competitive aspects of the grass-legume association. Advances in Agronomy, 33, 227–261.

Hill, N. S., Belesky, D. P., & Stringer, W. C. (1991). Competitiveness of tall fescue as influenced by Acremonium coenophialum. Crop Science, 31, 185–190.

Hill, N. S., Rottinghaus, G. E., Agee, C. S., & Schultz, L. M. (1993). Simplified sample preparation for HPLC analysis of ergovaline in tall fescue. Crop Science, 33, 331–333.

Jensen, T. L., Provenza, F. D., & Villalba, J. J. (2014). Influence of drinking water with quebracho tannin on intake of endophyte-infected tall fescue by cattle. Animal Feed Science and Technology, 188, 13–16.

Lee, S. T., Stegelmeier, B. L., & Gardner, D. R. (2001). The isolation and identification of steroidal sapogenins in switchgrass. Journal of Natural Toxins, 10(4), 273–281.

Lyman, T. D., Provenza, F. D., & Villalba, J. J. (2008). Sheep foraging behavior in response to interactions among alkaloids, tannins and saponins. Journal of the Science of Food and Agriculture, 88(5), 824–831.

Lyman, T. D., Provenza, F. D., Villalba, J. J., & Wiedmeier, R. D. (2011). Cattle preferences differ when endophyte-infected tall fescue, birdsfoot trefoil, and alfalfa are grazed in different sequences. Journal of Animal Science, 89(4), 1131–1137.

MacAdam, J. W., Griggs, T. C., Beuselinck, P. R., & Grabber, J. H. (2006). Birdsfoot trefoil, a valuable tannin-containing legume for mixed pastures. Online. Forage and grazinglands, September issue, 1–8. https://doi.org/10.1094/fg-2006-0912-01-rv

Mallarino, A. P., Wedin, W. F., Goyenola, R. S., Perdomo, C. H., & West, C. P. (1990). Legume species and proportion effects on symbiotic dinitrogen fixation in legume-grass mixtures. Agronomy Journal, 82, 785–789.

Mantz, G. K., Villalba, J. J., & Provenza, F. D. (2008). Supplemental polyethylene glycol affects intake of and preference for Sericea lespedeza by cattle. Journal of Animal Science, 87(2), 761–769.

Meuret, M. (1997). How do I cope with that bush?: Optimizing on less palatable feeds at pasture using the Menu model. Recent Advances in Small Ruminant Nutrition, 53–57.

Meuret, M., & Provenza, F. (2015). How French shepherds create meal sequences to stimulate intake and optimise use of forage diversity on rangeland. Animal Production Science, 55(3), 309.

Moore, K. J., & Moser, L. E. (1995). Quantifying developmental morphology of perennial grasses. Crop Science, 35(1), 37–43.

Moore, K. J., Moser, L. E., Vogel, K. P., Waller, S. S., Johnson, B. E., & Pedersen, J. F. (1991). Describing and quantifying growth stages of perennial forage grasses. Agronomy Journal, 83(6), 1073–1077.

Mueller-Harvey, I. (2006). Unravelling the conundrum of tannins in animal nutrition and health. Journal of the Science of Food and Agriculture, 86(13), 2010–2037.

Okuda, T., Mori, K., & Shiota, M. (1982). Effects of interaction of tannins and coexisting substances. III Formation and solubilization of precipitates with alkaloids. Journal of the Pharmaceutical Society of Japan, 102, 854–858.

Owens, J., Provenza, F. D., Wiedmeier, R. D., & Villalba, J. J. (2012a). Influence of saponins and tannins on intake and nutrient digestion of alkaloid-containing foods. Journal of the Science of Food and Agriculture, 92(11), 2373–2378. https://doi.org/10.1002/jsfa.5643

Owens, J., Provenza, F. D., Wiedmeier, R. D., & Villalba, J. J. (2012b). Supplementing endophyte-infected tall fescue or reed canarygrass with alfalfa or birdsfoot trefoil increases forage intake and digestibility by sheep. Journal of the Science of Food and Agriculture, 92(4), 987–992.

Parsons, A. J., Newman, J. A., Penning, P. D., Harvey, A., & Orr, R. J. (1994). Diet preference of sheep: effects of recent diet, physiological state and species abundance. Journal of Animal Ecology, 63(2), 465. https://doi.org/10.2307/5563

Patamalai, B., Hejtmancik, E., Bridges, C. H., Hill, D. W., & Camp, B. J. (1990). The isolation and identification of steroidal sapogenins in kleingrass. Veterinary and Human Toxicology, 32(4), 314–318.

Paterson, J., Forcherio, C., Larson, B., Samford, M., & Kerley, M. (1995). The effects of fescue toxicosis on beef cattle productivity. Journal of Animal Science, 73(3), 889–898.

Provenza, F. D., Ortega-Reyes, L., Scott, C. B., Lynch, J. J., & Burritt, E. A. (1994). Antiemetic drugs attenuate food aversions in sheep. Journal of Animal Science, 72(8), 1989–1994.

Rasmussen, S., Parsons, A. J., Xue, H., & Newman, J. A. (2007). High nutrient supply

and carbohydrate content reduce endophyte and alkaloid concentration. In The 6th

International Symposium on Fungal Endophytes of Grasses. Grassland Research

and Practice Series (pp. 141–146). Retrieved from

http://www.grassland.org.nz/publications/nzgrassland_publication_2404.pdf

Reed, J. D. (1986). Relationships among soluble phenolics, insoluble proanthocyanidins

and fiber in East African browse species. Journal of Range Management, 39(1), 5.

Rhodes, M. T., Paterson, J. A., Kerley, M. S., Garner, H. E., & Laughlin, M. H. (1991).

Reduced blood flow to peripheral and core body tissues in sheep and cattle

induced by endophyte-infected tall fescue. Journal of Animal Science, 69, 2033–

2043.

Rottinghaus, G. E., Garner, G. B., Cornell, C. N., & Ellis, J. L. (1991). HPLC method for

quantitating ergovaline in endophyte-infested tall fescue: Seasonal variation of

ergovaline levels in stems with leaf sheaths, leaf blades, and seed heads. Journal

of Agricultural and Food Chemistry, 39 (1), 112–115.

Sleugh, B., Moore, K. J., George, J. R., & Brummer, E. C. (2000). Binary legume–grass

mixtures improve forage yield, quality, and seasonal distribution. Agronomy

Journal, 92(1), 24–29.

Stidham, W. D., Brown, C. J., Daniels, L. B., Piper, E. L., & Fetherstone, H. E. (1982).

Toxic fescue linked to reduced milk output in ewes. Arkansas Farm Research,

31(9), 6.

Stuedemann, J. A., Wilkinson, S. R., Belesky, D. P., Devine, O. J., Breedlove, D. L.,

Thompson, F. N., . . . Townsend, W. E. (1985). Utilization and management of

endophyte-infected tall fescue: Effects on steer performance and behavior. P. 17–20. In J. D. Miller (Ed.), Proc. 41st South Pasture Forage Crop Improvement Conference, Raleigh, N.C. May 20-22. USDA/ARS Government Print Office, Washington, DC.

Suter, M., Connolly, J., Finn, J. A., Loges, R., Kirwan, L., Sebasti_a, M.-T., & Lüscher, A. (2015). Nitrogen yield advantage from grass-legume mixtures is robust over a wide range of legume proportions and environmental conditions. Global Change Biology, 21(6), 2424–2438.

Terrill, T. H., Rowan, A. M., Douglas, G. B., & Barry, T. N. (1992). Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. Journal of the Science of Food and Agriculture, 58(3), 321–329.

Thompson, F. N., Stuedemann, J. A., & Hill, N. S. (2001). Anti-quality factors associated with alkaloids in eastern temperate pasture. Journal of Range Management, 54(4), 474. https://doi.org/10.2307/4003119

Villalba, J. J., Spackman, C., Goff, B. M., Klotz, J. L., Griggs, T., & MacAdam, J. W. (2016). Interaction between a tannin-containing legume and endophyte-infected tall fescue seed on lambs' feeding behavior and physiology. Journal of Animal Science, 94(2), 845–857.

Wall, M. E., Eddy, C. R., McClennan, M. L., & Klumpp, M. E. (1952). Detection and estimation of steroidal sapogenins in plant tissue. Analytical Chemistry, 24(8), 1337–1341.

Wen, L., Roberts, C. A., Williams, J. E., Kallenbach, R. L., Beuselinck, P. R., & McGraw, R. L. (2003). Condensed tannin concentration of rhizomatous and nonrhizomatous birdsfoot trefoil in grazed mixtures and monocultures. Crop Science, 43(1), 302–306.

Wink, M. (2010). Functions and biotechnology of plant secondary metabolites. Chichester, West Sussex, UK: Wiley-Blackwell.

Xie, D.-Y., & Dixon, R. A. (2005). Proanthocyanidin biosynthesis – still more questions than answers? Phytochemistry, 66(18), 2127–2144.

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% H_2NCONH_2) 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 (Cheeke, 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 $CHCl_3$ (chloroform):distilled water mix (10 mL) (1X) and $CHCl_3$ (5 mL) (3X), discarding $CHCl_3$ layer each time. The aqueous extract was then extracted with water-saturated $C_4H_{10}O$ (n-butanol, 5 mL) (3X). The $C_4H_{10}O$ 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 ($R^2 = 0.98$).

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 C_3H_6O (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 C_3H_6O extracts were combined and solvent (100 µL) was placed in 16 mL screwcap test tubes in triplicates with a 95:5 $C_4H_{10}O$: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 $CHCl_3$ (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 $C_3H_6O:CHCl_3$ 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 $CHCl_3$ (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%

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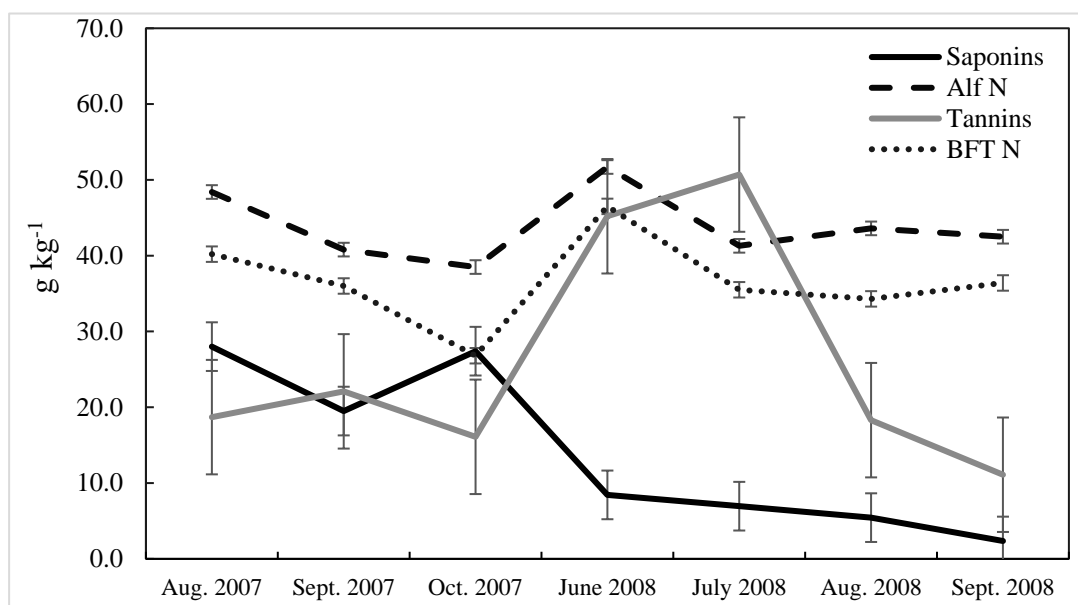
Fig. 3-1. Saponin means in alfalfa (g kg^{-1}) over time (P < 10^{-4}), tannin means in birdsfoot trefoil (g kg^{-1}) over time (P = 0.003), and total N means in Alf and BFT (g kg^{-1}) over time (both P < 10^{-4}) are shown (and SE) for 7 collection periods from 2007-2008.

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 g kg^{-1}), urea (36.7 g kg^{-1}), or unfertilized (36.1 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 µg kg^{-1}), urea (297 µg kg^{-1}), or unfertilized (222 µg kg^{-1}). Ergovaline concentration and total N in E+TF differed over time (P < 0.0001; Fig. 3-2). Total N means in E+TF were greater (P = 0.005; SE = 0.92) in plots fertilized with urea (33.4 g kg^{-1}) than in plots fertilized with feces (28.5 g kg^{-1}) or unfertilized plots (29.2 g kg^{-1}; Fig. 3-2).
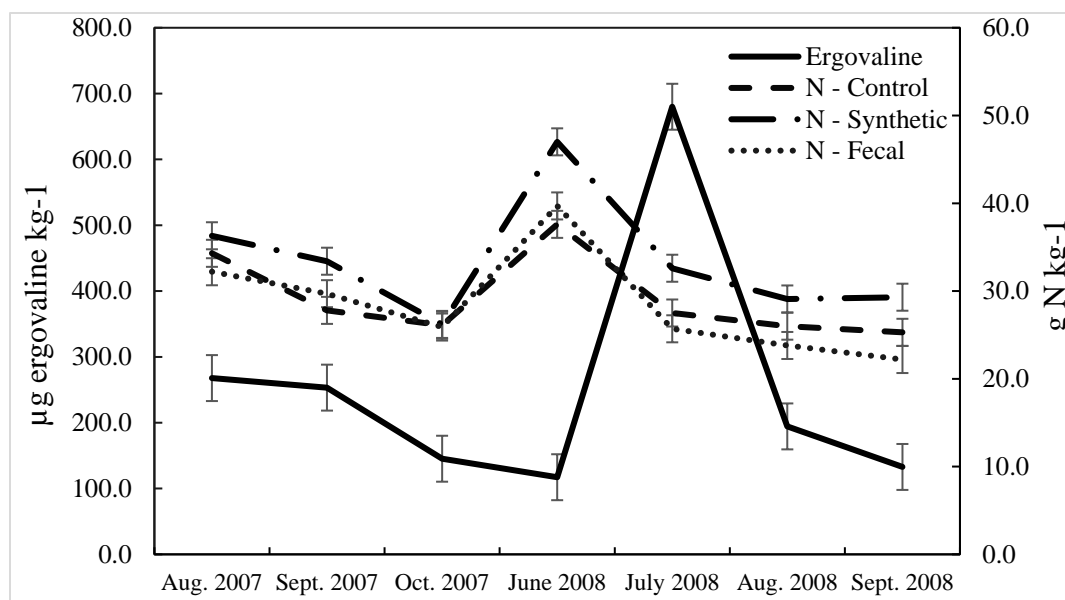
Fig. 3-2. Ergovaline means (µg kg^{-1}) in endophyte-infected tall fescue are shown over time (P < 10^{-4}). Total N (g kg^{-1}) treatment means (P = 0.005) in endophyte-infected tall fescue fertilized with feces (fecal), urea (synthetic), and without fertilizer (control) are shown over time (P < 10^{-4}) 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).
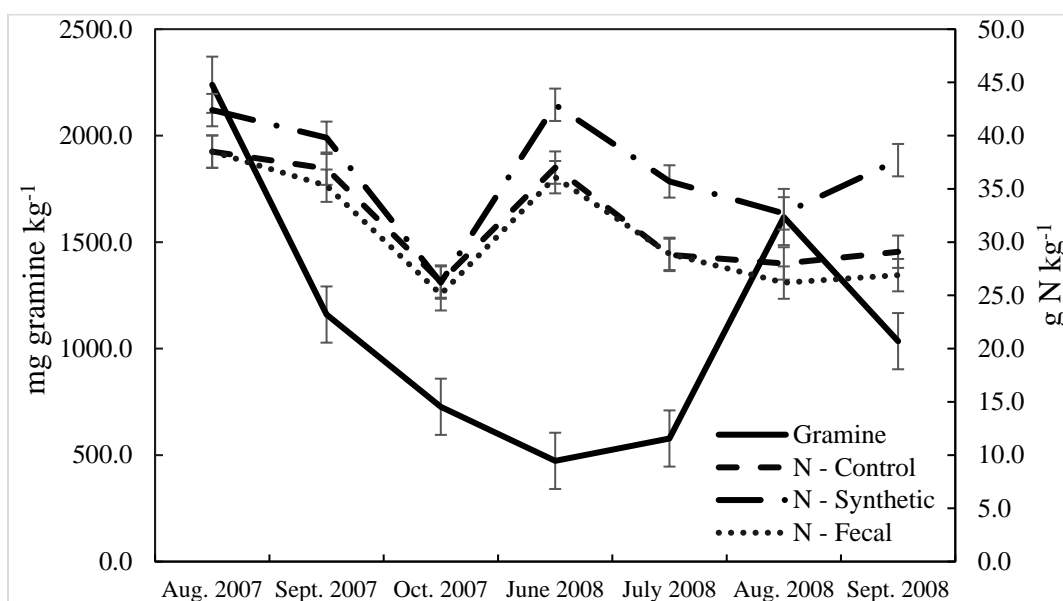
Fig. 3-3. Gramine means (mg kg^{-1}) in reed canarygrass are shown over time (P < 10^{-4}). Total N (g kg^{-1}) treatment means (P = 0.004) in reed canarygrass fertilized with feces (fecal), urea (synthetic), and without fertilizer (control) are shown over time (P < 10^{-4}) for 7 collection periods from 2007-2008.

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 adjacently 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 (NH_4NO_3) 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

Anderton, N., P.A. Cockrum, S.M. Colegate, J.A. Edgard, and K. Flower. 1999. Assessment of potential for toxicity of *Phalaris* spp. via alkaloid content determination: *P. coerulescens*, a case example. Phytochemical Analysis 10:113-118.

Arechavaleta, M., C. W. Bacon, R. D. Plattner, C. S. Hoveland, and D. E. Radcliffe. 1992. Accumulation of ergopeptide alkaloids in symbiotic tall fescue grown under deficits of soil water and nitrogen fertilizer. Applied and Environmental Microbiology 58(3):857–861.

Atsatt, P.R., and D.J. O'Dowd. 1976. Plant Defense Guilds. Science 193(4247):24–29.

Beauchamp, E.G. and J.W. Paul. 1989. A simple model to predict manure nitrogen availability to crops in the field. In: J.A. Hansen and K. Henrikson, editors, Nitrogen in organic wastes applied to soils. Academic Press, London. 140-149.

Berenbaum, M.R. 1995. The chemistry of defense: Theory and practice. Proceedings of the National Academy of Sciences 92(1):2–8.

Bottomley, P.J., and D.D. Myrold. 2007. Biological N inputs. In: E.A. Paul, Soil microbiology, ecology and biochemistry (Third Edition). Academic Press, San Diego, California. 365-387.

Bryant, J.P., S.F. Chapin, and D.R. Klein. 1983. Carbon / nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos, Copenhagen 40:357-368.

Bryant J.P., J. Tuomi and P. Niemela. 1988. Environmental constraint of constitutive and long-term inducible defenses in woody plants. In: K.C. Spencer, editor, Chemical mediation of coevolution. Academic Press, San Diego, California. 367–389.

Chambers, B.J., E.I. Lord, F.A. Nicholson, and K.A. Smith. 1999. Predicting nitrogen availability and losses following application of organic manures to arable land: MANNER. Soil use and management 15:137-143.

Chambers, B.J., K.A. Smith and T.J. Van Der Weerden. 1997. Ammonia emissions following the land spreading of solid manures. In: S.C. Jarvis and B.F. Pain, editors, Gaseous nitrogen emissions from grasslands. CAB Internat, Oxon, UK. 275-280.

Cheeke, P.R. 1998. Natural toxicants in feeds, forages, and poisonous plants - Second Edition. Interstate Publishers, Inc. Danville, Illinois.

Chichester, F.W. and R.F. Jr. Chaison. 1992. Analysis of carbon in calcareous soils using a two temperature dry combustion infrared instrumental procedure. Soil Science 153(3):237-241.

Clemensen, A.K., G.E. Rottinghaus, S.T. Lee, F.D. Provenza, and J.J. Villalba. 2018. How planting configuration influences plant secondary metabolites in tall fescue (*Festuca arundinacea* Schreb.), alfalfa (*Medicago sativa* L.), and birdsfoot trefoil (*Lotus corniculatus* L.): implications for grazing management. Grass and Forage Science 73:94-100.

Coley, P.D., J.P. Bryant, and F.S. Chapin. 1985. Resource availability and plant antiherbivore defense. Science 230(4728):895–99. doi:10.1126/science.230.4728.895.

Culvenor, C. C. J., R. Dal Bon, and L.W. Smith. 1964. The occurrence of indolealkylamine alkaloids in *Phalaris tuberosa L.* and *P. arundinacea L.* Australian Journal of Chemistry 17(11):1301–1304.

Danso, S.K.A., S. Curbelo, C. Labandera, and D. Pastorini. 1991. Herbage yield and nitrogen-fixation in a triple-species mixed sward of white clover, lotus and fescue. Soil Biology and Biochemistry 23(1):65–70. doi:10.1016/0038-0717(91)90163-E.

Feeny, P. 1976. Plant apparency and chemical defense. Recent Advances in Phytochemistry 10:1-40.

Flick, G.W., and S.C. Mueller. 1989. Alfalfa: Quality, maturity, and mean stage of development. Information bulletin 217. Department of Agronomy, College of Agriculture and Life Sciences, Cornell University Cooperative Extension, Ithaca NY.

Friend, M. A., F.D. Provenza, and J.J. Villalba. 2015. Preference by sheep for endophyte-infected tall fescue grown adjacent to or at a distance from alfalfa. Animal 9(3):516–525. doi:10.1017/S1751731114002559.

Gavlak, R., D. Horneck, R.O. Miller, and J. Kotuby-Amacher. 2003 Soil, plant and water reference methods for the western region. 2nd ed. WCC-103 Publ. Colorado State Univ., Ft. Collins.

Gentry, C.E., R.A. Chapman, L. Henson, and R.C. Bucknter. 1969. Factors affecting the alkaloid content of tall fescue (*Festuca arundinacea* Schreb.). Agronomy Journal 61:313-316.

Hartmann, T. 2007. From waste products to ecochemicals: Fifty years research of plant secondary metabolism. Phytochemistry 68(22–24):2831–2846. doi:10.1016/j.phytochem.2007.09.017.

Haynes, R.J. 1980. Competitive aspects of the grass-legume association. Advances in Agronomy 33:227–261.

Herms, D.A., and W.J. Mattson. 1992. The dilemma of plants: To grow or defend. The
    Quarterly Review of Biology. 67(3).

Hill, N.S., W.C. Stringer, G.E. Rottinghaus, D.P. Belesky, W.A. Parrott, and D.D. Pope.
    1990. Growth, morphological, and chemical component responses of tall fescue to
    Acremonium coenophialum. Crop Science 30:156-161.

Hill, N.S., D.P. Belesky, and W.C. Stringer. 1991. Competitiveness of tall fescue as
    influenced by *Acremonium coenophialum*. Crop Science 31:185-190.

Hill, N.S., G.E. Rottinghaus, C.S. Agee, and L.M. Schultz. 1993. Simplified sample
    preparation for HPLC analysis of ergovaline in tall fescue. Crop Science 33:331-
    333.

Hofland-Zijlstra, J.D., and F. Berendse. 2009. The effect of nutrient supply and light
    intensity on tannins and mycorrhizal colonisation in Dutch heathland ecosystems.
    Plant Ecology 201(2):661–75. doi:10.1007/s11258-008-9554-3.

Jarrell, W.M. and R.B. Beverly. 1981. The dilution effect in plant nutrition studies.
    Advances in Agronomy 34:197-224.

Jones, C.G., and R.D. Firn. 1991. On the evolution of plant secondary chemical diversity.
    Philosophical Transactions of the Royal Society of London B 333:273–280.

Ju, H.-J., N.S. Hill, T. Abbott, and K.T. Ingram. 2006. Temperature influences on
    endophyte growth in tall fescue. Crop Science 46(1):404–412.
    doi:10.2135/cropsci2005.0282.

Koenig, R., C. Hurst, J. Barnhill, B. Kitchen, M. Winger, and M. Johnson. 1999.
    Fertilizer management for alfalfa. All Archived Publications. Paper 7.
    http://digitalcommons.usu.edu/extension_histall/7

Koenig, R.T., M. Nelson, J. Barnhill, and D. Miner. 2002. Fertilizer management for grass and grass-legume mixtures. All Archived Publications. Paper 9. http://digitalcommons.usu.edu/extension_histall/9

Koricheva, J. 2002. The carbon-nutrient balance hypothesis is dead; long live the carbon-nutrient balance hypothesis? Oikos 98(3):537–539.

Kumar, K., S.C. Gupta, S.K. Baidoo, Y. Chander, and C.J. Rosen. 2005. Antibiotic uptake by plants from soil fertilized with animal manure. Journal of Environmental Quality. 34(6):2082–2085.

Lee, S.T., K.P. Vogel, D.R. Gardner, and B.L. Stegelmeier. 2001. The isolation and identification of steroidal sapogenins in switchgrass. Journal of Natural Toxins 10(4):273–81.

Loomis, W. E. 1932. Growth-differentiation balance vs. carbohydrate-nitrogen ratio. Proceedings of the American Society for Horticultural Science 29:240-245.

Lu, C.D. and N.A. Jorgensen. 1987. Alfalfa saponins affect site and extent of nutrient digestion in ruminants. Journal of Nutrition 117(5):919-927.

Majak, W., R.E. McDiarmid, T.W. Powell, A.L. Van Ryswyk, D.G. Stout, R.J. Williams, and R.E. Tucker. 1979. Relationships between alkaloids in reed canarygrass (*Phalaris arundinacea*), soil moisture and nitrogen fertility. Plant, Cell & Environment 2(4):335–40.

Mallarino, A.P., C.H. Perdomo, C.P. West, W.F. Wedin, and R.S. Goyenola. 1990. Legume species and proportion effects on symbiotic dinitrogen fixation in legume-grass mixtures. Agronomy Journal 82(4):785–89.

Mantz, G. K., J.J. Villalba, and F.D. Provenza. 2008. Supplemental polyethylene glycol affects intake of and preference for sericea lespedeza by cattle. Journal of Animal Science 87(2):761–69. doi:10.2527/jas.2007-0706.

Marten, G.C. 1973. Alkaloids in reed canarygrass. In: A.G. Matches et al., editors, Anti-quality components of forages. CSSA Spec. Publ. 4. CSSA, Madison, WI. p. 15-31.

Marten, G.C., C.E. Clapp, and W.E. Larson. 1979. Effects of municipal wastewater effluent and cutting management on persistence and yield of eight perennial forages. Agronomy Journal 71:650-658.

McKey, D. 1974. Adaptive patterns in alkaloid physiology. American Naturalist 108(961):305–320.

Moore, K.J., L.E. Moser, K.P. Vogel, S.S. Waller, B.E. Johnson, and J.F. Pedersen. 1991. Describing and quantifying growth stages of perennial forage grasses. Agronomy Journal 83:1073-1077.

Moore, K.J., and L.E. Moser. 1995. Quantifying developmental morphology of perennial grasses. Crop Science 35:37-43.

Palumbo, M.J., F.E. Putz, and S.T. Talcott. 2007. Nitrogen fertilizer and gender effects on the secondary metabolism of yaupon, a caffeine-containing North American holly. Oecologia 151(1):1–9.

Patamalai, B., D.W. Hill, B.J. Camp, E. Hejtmancik, and C.H. Bridges. 1990. The isolation and identification of steroidal sapogenins in kleingrass. Veterinary and Human Toxicology 32(4):314–318.

Porter, J.K. 1994. Chemical constituents of grass endophytes. In: C.W. Bacon and J.F. White, Jr., Biotechnology of endophytic fungi of grasses. CRC Press, Boca Raton, Florida. 103-123.

Powers, J.C., and I. Ponticello. 1968. A total synthesis of the diazaphenanthrene alkaloid perlolidine. Journal of the American Chemical Society 90:7102-7106.

Price, P.W. 1991. Darwinian methodology and the theory of insect herbivore population dynamics. Annals of the Entomological Society of America 84(5):465–473.

Reed, J.D. 1986. Relationships among soluble phenolics, insoluble proanthocyanidins and fiber in East African browse species. Journal of Range Management 39(1):5-7. doi:10.2307/3899675.

Rhoades, D.F. and R.G. Cates. 1976. Towards a general theory of plant antiherbivore chemistry. Recent Advancements in Phytochemistry 10:168-213.

Rhoades, D.F. 1979. Evolution of plant chemical defense against herbivores. In: G. A. Rosenthal, and D. H. Janzen, editors, Herbivores, their interaction with secondary plant metabolites. Academic Press, New York. 3-54.

Rottinghaus, G.E., G.B. Garner, C.N. Cornell, and J.L. Ellis. 1991. HPLC Method for quantitating ergovaline in endophyte-infested tall fescue: Seasonal variation of ergovaline levels in stems with leaf sheaths, leaf blades, and seed heads. Journal of Agricultural and Food Chemistry 39(1):112–115.

Szakiel, A., C. Pączkowski, and M. Henry. 2011. Influence of environmental abiotic factors on the content of saponins in plants. Phytochemistry Reviews 10(4):471–491. doi:10.1007/s11101-010-9177-x.

Tava, A., M. Odoardi, and W. Oleszek. 1999. Seasonal changes of saponin content in five alfalfa (*Medicago sativa*) cultivars. Agricoltura Mediterranea 129(2/3):111–16.

Terrill, T H, A.M Rowan, G.B. Douglas, and T.N. Barry. 1992. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. Journal of the Science of Food and Agriculture 58(3):321–29. doi:10.1002/jsfa.2740580306.

Thompson, F.N., J.A. Stuedemann, and N.S. Hill. 2001. Anti-quality factors associated with alkaloids in eastern temperate pasture. Journal of Range Management 54(4):474. doi:10.2307/4003119.

Tuomi, J., P. Niemelä, E. Haukioja, S. Sirén, and S. Neuvonen. 1984. Nutrient stress: An explanation for plant anti-herbivore responses to defoliation. Oecologia 61(2):208–210.

Verma, N., and S. Shukla. 2015. Impact of various factors responsible for fluctuation in plant secondary metabolites. Journal of Applied Research on Medicinal and Aromatic Plants 2(4):105–113. doi:10.1016/j.jarmap.2015.09.002.

Vijay, N., A. Kumar, and A. Bhoite. 2009. Influence of nitrogen, phosphorus and potassium fertilizer on biochemical contents of *Asparagus racemosus* (Willd.) root tubers. Research Journal of Environmental Sciences 3(3):285–291.

Wall, M.E., C.R. Eddy, M.L. McClennan, and M.E. Klumpp. 1952. Detection and
    estimation of steroidal sapogenins in plant tissue. Analytical Chemistry
    24(8):1337–1341.

Wilkinson, S. 1958. 5-Methoxy-N-Methyltryptamine: A new indole alkaloid from
    Phalaris arundinacea L. Journal of the Chemical Society 2079–2081.
    doi:10.1039/JR9580002079.

Zhu, Z., Z. Liang, R. Han, and X. Wang. 2009. Impact of fertilization on drought
    response in the medicinal herb *Bupleurum chinense* DC.: Growth and
    saikosaponin production. Industrial Crops and Products 29(2–3):629–633.
    doi:10.1016/j.indcrop.2008.08.002.

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 over-winter 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 (NO_3^- -N), using the Cadmium Reduction method (Method S-3.10), and ammonium (NH_4^+ -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 1.3g cm$^{3\,-1}$.

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 1.3g cm$^{3\,-1}$. 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 NO_3^-, NH_4^+, 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:

$$\frac{3.74 \; kg \; DM \; manure}{cow \; per \; day} \times \frac{12 \; cows}{12{,}285.75 \; m^2} \times \frac{10{,}000 m^2}{ha} \times \frac{81 \; days}{1}$$

$$= \frac{2959.18 \; kg \; DM \; manure}{ha}$$

Equation 2:

$$\frac{2959.18 \; kg \; DM \; manure}{ha} \times \frac{ha \; furrow \; slice}{1500 \; m^3} \times \frac{m^3}{1{,}000{,}000 \; cm^3} \times \frac{cm^3}{1.3 \; g \; soil} \times \frac{1000 \; g}{kg}$$

$$\times \frac{500 \; g \; soil}{bag} = \frac{0.76 \; g \; manure}{bag}$$

Feces rate based on N content:

Equation 1:

$$\frac{350 \; kg \; N}{hafs} \times \frac{hafs}{1500 m^3} \times \frac{m^3}{1{,}000{,}000 \; cm^3} \times \frac{cm^3}{1.3 \; g \; soil} \times \frac{1000 \; g}{kg} \times \frac{100 \; kg \; DM}{2.14 \; kg \; N}$$

$$\times \frac{.500 \; kg \; soil}{bag} \times \frac{1000 \; g \; DM}{kg \; DM} = \frac{4.19 g \; DM \; manure}{bag}$$

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).

2012 Grazed Study					
	Legume (SE)		*Planting Configuration (SE)*		
Soil property	**Alfalfa**	**Sainfoin**	**TF Mono**	**Legume Mono**	**Mix**
g⁻¹ soil					
Ammonium, µg (0-30 cm depth)	0.48 (0.47)	0.21	0.33 (0.47)	0.00	0.75
Ammonium, µg (30-60 cm depth)	0.00 (0.38)	0.55	0.17 (0.38)	0.04	0.60
Ammonium, µg (60-90 cm depth)	0.00 (0.20)	0.17	0.00 (0.22)	0.04	0.19
Nitrate, µg (0-30 cm depth)	7.47 (0.53) *	1.76	1.54 (1.39)	8.06	4.25
Nitrate, µg (30-60 cm depth)	1.26 (0.36)	0.77	0.81 (0.32)	1.43	0.81
Nitrate, µg (60-90 cm depth)	0.50 (0.19)	0.22	0.00 (0.22)	0.87	0.25
Dehydrogenase, µg TPF (0-10 cm depth)	9.05 (0.74)	10.7	10.3 (0.80)	9.86	9.5
Dehydrogenase, µg TPF (10-20 cm depth)	4.57 (0.49)	4.93	5.43 (0.50) * a	4.34 b	4.50 ab
Dehydrogenase, µg TPF (20-30 cm depth)	3.35 (0.43)	3.44	3.70 (0.46)	3.24	3.24
Total N, mg	1.22 (0.03)	1.24	1.23 (0.04)	1.25	1.20
Organic C, mg	7.68 (0.42)	7.80	7.82 (0.48) * ab	7.12 b	8.28 a
Olsen P, µg	8.43 (1.41)	8.73	7.77 (1.40) * a	10.20 b	7.80 a
Olsen K, µg	168 (19.0)	158	199 (17.2) ** a	140 b	149 b
Zinc, µg	1.39 (0.21)	1.35	1.40 (0.23)	1.43	1.27
Iron, µg	10.2 (0.70)	9.77	10.7 (0.73)	9.86	9.37
Copper, µg	1.49 (0.44)	1.24	1.32 (0.44)	1.49	1.30
Manganese, µg	9.82 (0.69)	9.70	10.1 (0.68)	9.74	9.40
Phenol Oxidase, µg Dopachrome	1.36 (0.06)	1.35	1.28 (0.06) * b	1.44 a	1.35 ab
Microbial respiration, µg	3.34 (0.31)	3.94	4.41 (0.28) ** a	2.96 b	3.56 ab
Microbial biomass, µg	646 (50.5)	649	649 (52.7)	668	625
Readily Mineralizable C, µg	24.5 (2.10)	24.4	28.0 (2.11) ** a	21.6 b	23.8 b
Microbial biomass C / Organic C	852 (60.1)	848	836 (70.6)	951	762
qCO₂ (Mic. respiration Mic. biomass⁻¹)	0.005 (.0008)	0.006	0.007 (.0007) * a	0.005 b	0.006 ab
Porosity, mg	5.07 (0.10)	5.04	5.18 (0.12)	4.97	5.03
Soil property					
C:N ratio	6.31 (0.38)	6.32	6.38 (0.40) ** a	5.69 b	6.89 a
pH	8.41 (0.03)	8.42	8.42 (0.03)	8.40	8.43
EC, dS m⁻¹	0.24 (0.03) *	0.20	0.22 (0.03)	0.21	0.24
Bulk Density, g cm³ ⁻¹	1.31 (0.03)	1.31	1.28 (0.03)	1.33	1.32
* Significant at p < 0.05					
** Significant at p < 0.01					

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

2012 Pregrazing Biomass t ha^{-1}		Period 1		Period 2		Period 3	
		4-Jun	9-Jul	23-Jul	22-Aug	30-Aug	13-Sep
Alfalfa	Legume	43.5 ± 0.3	63.2 ± 2.6	47.1 ± 4.2	42.6 ± 3.8	45.7 ± 3.3	41.9 ± 1.2
	Mix	32.8 ± 2.8	34.1 ± 2.1	23.0 ± 3.1	32.4 ± 0.5	30.4 ± 3.4	30.6 ± 4.1
	Fescue	36.9 ± 4.4	29.8 ± 4.2	25.3 ± 2.8	33.9 ± 2.0	32.6 ± 3.3	33.0 ± 2.5
Sainfoin	Legume	59.3 ± 9.3	107.3 ± 1.1	39.8 ± 4.3	63.9 ± 1.5	35.5 ± 6.7	27.1 ± 5.4
	Mix	31.5 ± 1.9	34.3 ± 5.0	22.7 ± 2.0	30.3 ± 1.8	27.1 ± 1.7	28.6 ± 1.3
	Fescue	36.7 ± 3.5	32.8 ± 3.6	22.1 ± 1.3	29.4 ± 0.5	28.5 ± 3.2	25.5 ± 1.8

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

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 (qCO_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 qCO_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.

2012 Grazed Study							
	Planting Config. (SE)		*Month (SE)*			*Legume (SE)*	
Plant property	**Mono**	**Mix**	**June**	**Aug.**	**Sept.**	**Alfalfa**	**Sainfoin**
Alfalfa							
Saponins, g kg^{-1}	4.63 (1.44)	6.78	7.21 (1.59)	6.28	3.64	-	-
Nitrogen, g kg^{-1}	46.3 (3.96)	44.3	43.7 (3.81)	48.7	43.5	-	-
Sainfoin							
Tannins, g kg^{-1}	58.4 (2.61)	59.3	60.6 (2.88) * a	61.1 a	54.9 b	-	-
Nitrogen, g kg^{-1}	40.4 (0.34)	39.8	41.0 (0.49) * a	38.3 b	41.0 a	-	-
Tall Fescue							
Nitrogen, g kg-1	27.9 (0.59)	26.0	28.6 (0.94)	27.0	25.2	28.3 (0.33) *	25.6
w/Alfalfa	29.0 (0.84)	27.5	30.1 (1.34)	28.3	26.5	-	-
w/Sainfoin	26.7 (0.84)	24.6	27.1 (1.34)	25.8	24.0	-	-
* 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 NO_3^- 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
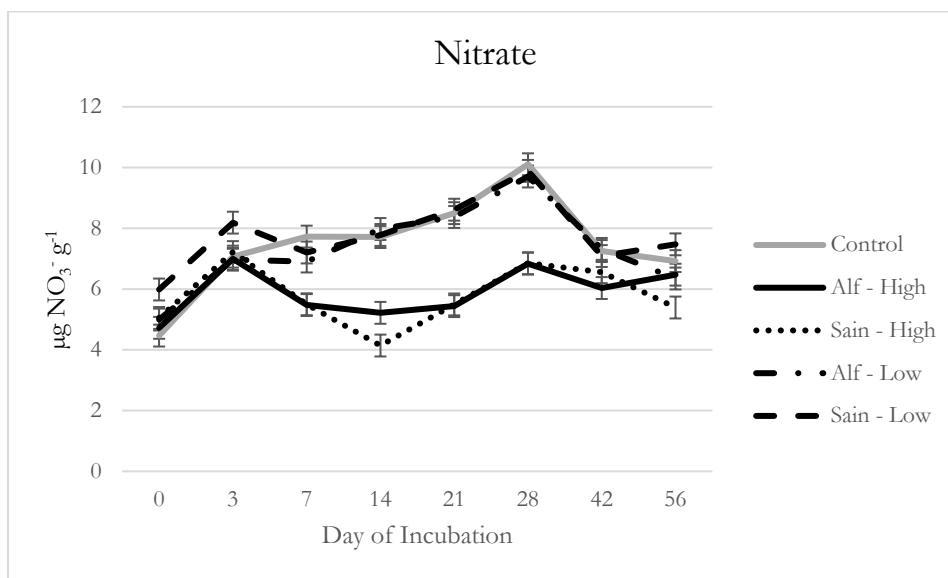
4:1.

Fig. 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.
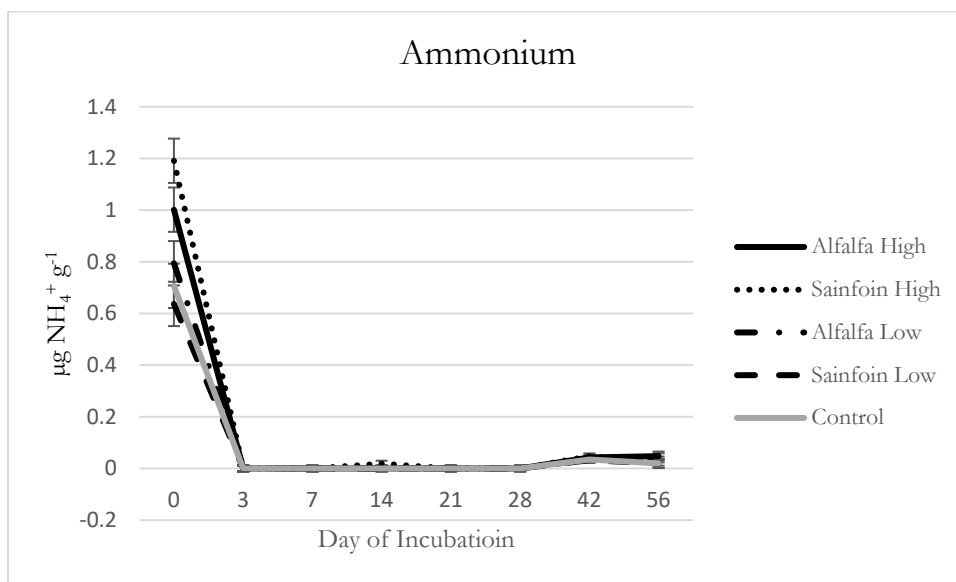
Fig. 4-2. Incubation study - ammonium results on two rates of feces (high / low) from two different diets of either alfalfa (Alf) or sainfoin (Sain) measured 8 times over 56 days.

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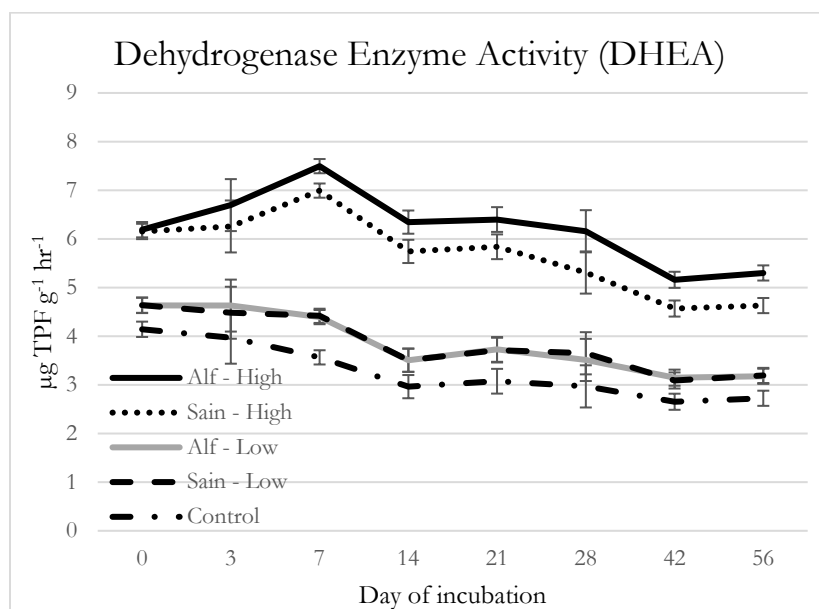
Fig. 4-3. Incubation study – dehydrogenase results on two rates of feces (high / low) from two different diets of either alfalfa (Alf) or sainfoin (Sain) measured 8 times over 56 days.

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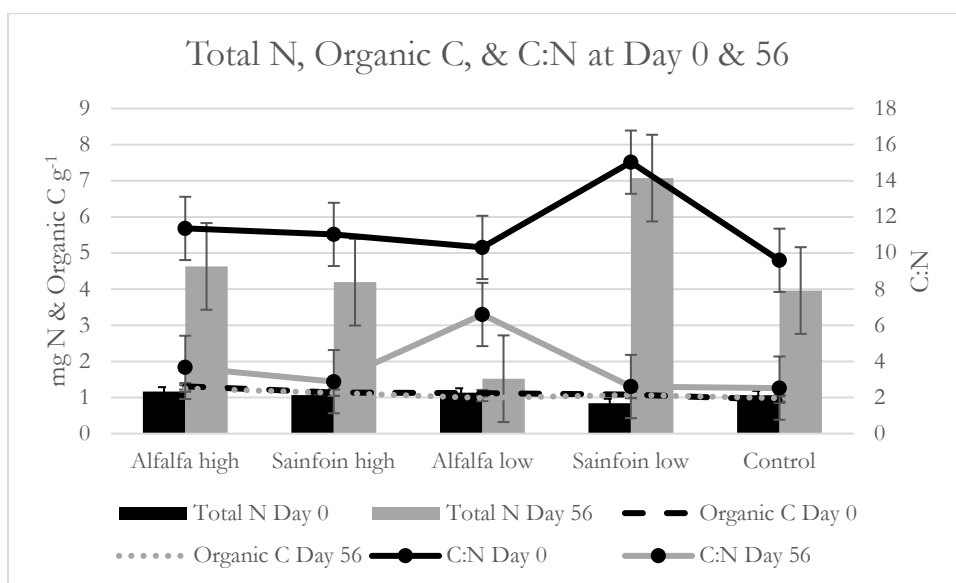
Fig. 4-4. Incubation study – total nitrogen, total organic carbon, and C:N ratio on two
rates of feces (high / low) from two different diets of either alfalfa or sainfoin measured
at the beginning and end of the study (days 0 and 56).

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.

References

Abbasi, M.K., Hina, M., Khalique, A., Khan, S.R. 2007. Mineralization of three organic manures used as nitrogen source in a soil incubated under laboratory conditions. Commun Soil Sci Plant Anal. 38, 1691-1711.

Adamczyk, B., Adamczyk, S., Smolander, A., Kitunen, V., 2011. Tannic acid and Norway spruce condensed tannins can precipitate various organic nitrogen

compounds. Soil Biol. Biochem. 43, 628–637.

https://doi.org/10.1016/j.soilbio.2010.11.034

Adamczyk, B., Kitunen, V., Smolander, A., 2009. Polyphenol oxidase, tannase and

proteolytic activity in relation to tannin concentration in the soil organic horizon

under silver birch and Norway spruce. Soil Biol. Biochem. 41, 2085–2093.

https://doi.org/10.1016/j.soilbio.2009.07.018

Adamczyk, S., Kiikkilä, O., Kitunen, V., Smolander, A., 2013. Potential response of soil

processes to diterpenes, triterpenes and tannins: Nitrification, growth of

microorganisms and precipitation of proteins. Appl. Soil Ecol. 67, 47–52.

https://doi.org/10.1016/j.apsoil.2013.02.009

AlSaadawi, I.S., 1988. Biological suppression of nitrification by selected cultivars of

Helianthus annum L. J. Chem. Ecol. 14, 733–741.

https://doi.org/10.1007/BF01018768

Anderson, J.P.E., Domsch, K.H., 1978. A physiological method for the quantitative

measurement of microbial biomass in soils. Soil Biol. Biochem. 10, 215–221.

Baitilwake, M.A, Salomez, J., Mrema, J.P, De Neve, S., 2012. Nitrogen mineralization of

two manures as influenced by contrasting application methods under laboratory

conditions. Commun. Soil Sci Plant Anal. 43: 1-2, 357-367.

http://dx.doi.org/10.1080/00103624.2012.641473

Barry, T.N., Manley T.R, Duncan S.J., 1986. The role of condensed tannins in the

nutritional value of Lotus pedunculatus for sheep. 4. Sites of carbohydrate and

protein digestion as influenced by dietary reactive tannin concentration. Br J Nutr.

55, 1, 123-37.

Barry, T.N., McNabb, W.C., 1999. The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. Br. J. Nutr. 81, 263–272.

Beauchemin, K.A., McGinn, S.M., Martinez, T.F., McAllister, T.A., 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. J. Anim. Sci. 85, 1990–1996. https://doi.org/10.2527/jas.2006-686

Blake, G.R., 1965. Bulk Density1, in: Methods of Soil Analysis. Part 1. Physical and Mineralogical Properties, Including Statistics of Measurement and Sampling, Agronomy Monograph. American Society of Agronomy, Soil Science Society of America, Madison, WI, pp. 374–390.

Boadi, D., Benchaar, C., Chiquette, J., Massé, D., 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: update review. Can. J. Anim. Sci. 84, 319–335.

Bradley, R.L., Titus, B.D., Preston, C.P., 2000. Changes to mineral N cycling and microbial communities in black spruce humus after additions of (NH 4) 2 SO 4 and condensed tannins extracted from Kalmia angustifolia and balsam fir. Soil Biol. Biochem. 32, 1227–1240.

Burgos, P., Madejón, E., Cabrera, F., 2002. Changes in soil organic matter, enzymatic activities and heavy metal availability induced by application of organic residues, in: Violante, A., Huang, P.M., Bollag, J.-M., Gianfreda, L. (Eds.), Developments in Soil Science. Elsevier, pp. 353–362. https://doi.org/10.1016/S0166-2481(02)80030-7

Cadisch, G., Schunke, R.M., Giller, K.E., 1994. Nitrogen cycling in a pure grass pasture and a grass-legume mixture on a red latosol in Brazil. Trop. Grassl. 28, 43-52.

Cheeke, P.R., 1998. Natural toxicants in feeds, forages, and poisonous plants / by Peter R. Cheeke. Danville, Ill. : Interstate Publishers.

Chellemi, D.O., Porter, I.J., 2001. The role of plant pathology in understanding soil health and its application to production agriculture. Australas. Plant Pathol. 30, 103–109.

Chichester, F.W., Chaison, R.F. Jr., 1992. Analysis of carbon in calcareous soils using a two temperature dry combustion infrared instrumental procedure. Soil Sci. 153: 3, 237-241.

Chu, H. Lin, X., Fujii, T. Morimoto, S., Yagi, K., Hu, J., Zhang, J., 2007. Soil microbial biomass, dehydrogenase activity, bacterial community structure in response to long-term fertilizer management. Soil Biol. Biochem. 39, 11, 2971-2976.

Clemensen, A.K., Provenza, F.D., Lee, S.T., Gardner, D.R., Rottinghaus, G.E., Villalba, J.J., 2017. Plant Secondary Metabolites in Alfalfa, Birdsfoot Trefoil, Reed Canarygrass, and Tall Fescue Unaffected by Two Different Nitrogen Sources. Crop Sci. 57, 964-970. https://doi.org/10.2135/cropsci2016.08.0680

Clemensen, A.K., Rottinghaus, G.E., Lee, S.T., Provenza, F.D., Villalba, J.J., 2018. How planting configuration influences plant secondary metabolites in tall fescue (*Festuca arundinacea* Schreb.), alfalfa (*Medicago sativa* L.), and birdsfoot trefoil (*Lotus corniculatus* L.): implications for grazing management. Grass Forage Sci. 73:94-100.

Cooper, J.M., Warman, P.R., 1997. Effects of three fertility amendments on soil dehydrogenase activity, organic C and pH. Can. J. Soil Sci. 77, 281-283.

Davidson, E.A., Galloway, L.F., Strand, M.K., 1987. Assessing available carbon: Comparison of techniques across selected forest soils. Commun. Soil Sci. Plant Anal. 18, 45–64. https://doi.org/10.1080/00103628709367802

Delve, R.J., Cadisch, G., Tanner, J.C., Thorpe, W., Thorne, P.J., Giller, K.E., 2001. Implications of livestock feeding management on soil fertility in the smallholder farming systems of sub-Saharan Africa. Agric. Ecosyst. Environ. 84, 227-243.

Di, H.J., Cameron, K.C., 2002. Nitrate leaching in temperate agroecosystems: sources, factors and mitigating strategies. Nutr. Cycl. Agroecosystems 64, 237–256. https://doi.org/10.1023/A:10214715311884

Eisenhauer, N., Beβler, H., Engels, C., Gleixner, G., Habekost, M., Milcu, A., Partsch, S., Sabais, A.C.W., Scherber, C., Steinbeiss, S., Weigelt, A., Weisser, W.W., Scheu, S., 2010. Plant diversity effects on soil microorganisms support the singular hypothesis. Ecol. 91, 2, 485-496.

Flick, G.W., Mueller, S.C., 1989. Alfalfa: Quality, Maturity, and Mean Stage of Development. Information Bulletin 217. Department of Agronomy, College of Agriculture and Life Sciences, Cornell University Cooperative Extension, Ithaca NY.

Franzluebbers, A.J., 2006. Short-term Responses of Soil C and N Fractions to Tall Fescue Endophyte Infection. Plant Soil 282, 153–164. https://doi.org/10.1007/s11104-005-5447-6

Franzluebbers, A.J., Hill, N.S., 2005. Soil carbon, nitrogen, and ergot alkaloids with short-and long-term exposure to endophyte-infected and endophyte-free tall fescue. Soil Sci. Soc. Am. J. 69, 404–412.

Franzluebbers, A.J., Nazih, N., Stuedemann, J.A., Fuhrmann, J.J., Schomberg, H.H., Hartel, P.G., 1999. Soil Carbon and Nitrogen Pools under Low- and High-Endophyte-Infected Tall Fescue 1 Trade and company names are included for the benefit of the reader and do not imply any endorsement or preferential treatment of the product listed by the U.S. Department of Agriculture. Soil Sci. Soc. Am. J. 63, 1687–1694. https://doi.org/10.2136/sssaj1999.6361687x

Franzluebbers, A.J., Stuedemann, J.A., 2005. Soil carbon and nitrogen pools in response to tall fescue endophyte infection, fertilization, and cultivar. Soil Sci. Soc. Am. J. 69, 396–403.

Gavlak, R., Horneck, D., Miller, R.O., Kotuby-Amacher, J., 2003. Soil, plant and water reference methods for the western region. WCC-103 Publ. WREP-125 17–36.

Haynes, R.J., Williams, P.H., 1993. Nutrient Cycling and Soil Fertility in the Grazed Pasture Ecosystem, in: Sparks, D.L. (Ed.), Advances in Agronomy. Academic Press, pp. 119–199. https://doi.org/10.1016/S0065-2113(08)60794-4

Honeycutt, C.W., Griffin, T.S., Wienhold, B.J., Eghball, B., Albrecht, S.L., Powell, J.M., Woodbury, B.L., Sistani, K.R., Hubbard, R.K., Torbert, H.A., 2005. Protocols for Nationally Coordinated Laboratory and Field Research on Manure Nitrogen Mineralization. Commun. Soil Sci. Plant Anal. 36, 2807–2822. https://doi.org/10.1080/00103620500304184

Hooper, D.U., Vitousek, P., 1998. Effect of plant composition and diversity on nutrient cycling. Ecol. Monograph. 68, 121-149.

Joanisse, G.D., Bradley, R.L., Preston, C.M., Munson, A.D., 2007. Soil enzyme inhibition by condensed litter tannins may drive ecosystem structure and

processes: the case of Kalmia angustifolia. New Phytol. 175, 535–546. https://doi.org/10.1111/j.1469-8137.2007.02113.x

Kelleher, B.P., Simpson, M.J., Simpson, A.J., 2006. Assessing the fate and transformation of plant residues in the terrestrial environment using HR-MAS NMR spectroscopy. Geochim. Cosmochim. Acta 70, 4080–4094. https://doi.org/10.1016/j.gca.2006.06.012

Krall, J.M., Delaney, R.H., 1982. Assessment of Acetylene Reduction by Sainfoin and Alfalfa over Three Growing Seasons1. Crop Sci. 22, 762–766. https://doi.org/10.2135/cropsci1982.0011183X002200040015x

Kraus, T.E.C., Dahlgren, R.A., Zasoski, R.J., 2003. Tannins in nutrient dynamics of forest ecosystems - a review. Plant Soil 256, 41–66. https://doi.org/10.1023/A:1026206511084

LeBel, P., Thiffault, N., Bradley, R.L., 2008. Kalmia removal increases nutrient supply and growth of black spruce seedlings: An effect fertilizer cannot emulate. For. Ecol. Manag. 256, 1780–1784. https://doi.org/10.1016/j.foreco.2008.02.050

Lee, S.T., Vogel, K.P., Gardner, D.R., Stegelmeier, B.L., 2001. The isolation and identification of steroidal sapogenins in switchgrass [electronic resource]. J. Nat. Toxins 10, 273–281.

Lorenz, K., Preston, C.M., Raspe, S., Morrison, I.K., Feger, K.H., 2000. Litter decomposition and humus characteristics in Canadian and German spruce ecosystems: information from tannin analysis and< sup> 13</sup> C CPMAS NMR. Soil Biol. Biochem. 32, 779–792.

Madritch, M.D., Lindroth, R.L., 2015. Condensed tannins increase nitrogen recovery by trees following insect defoliation. New Phytol. 208, 410–420. https://doi.org/10.1111/nph.13444

Mantz, G.K., Villalba, J.J., Provenza, F.D., 2008. Supplemental polyethylene glycol affects intake of and preference for sericea lespedeza by cattle. J. Anim. Sci. 87, 761–769. https://doi.org/10.2527/jas.2007-0706

Maughan, B., Provenza, F.D., Tansawat, R., Maughan, C., Martini, S., Ward, R., Clemensen, A., Song, X., Cornforth, D., Villalba, J.J., 2014. Importance of grass-legume choices on cattle grazing behavior, performance and meat characteristics. J. Anim. Sci. 92, 2309.

Meuret, M., Provenza, F., 2015. How French shepherds create meal sequences to stimulate intake and optimise use of forage diversity on rangeland. Anim. Prod. Sci. 55, 309. https://doi.org/10.1071/AN14415

Moore, K.J., Moser, L.E., 1995. Quantifying developmental morphology of perennial grasses. Crop Sci. 35, 37–43.

Moore, K.J., Moser, L.E., Vogel, K.P., Waller, S.S., Johnson, B.E., Pedersen, J.F., 1991. Describing and quantifying growth stages of perennial forage grasses. Agron. J. 83, 1073–1077.

Nennich, T.D., Harrison, J.H., VanWieringen, L.M., Meyer, D., Heinrichs, A.J., Weiss, W.P., St-Pierre, N.R., Kincaid, R.L., Davidson, D.L., Block, E., 2005. Prediction of manure and nutrient excretion from dairy cattle. J. Dairy Sci. 88, 3721–3733.

Omacini, M., J Chaneton, E., M Ghersa, C., Otero, P., 2004. Do foliar endophytes affect grass litter decomposition? A microcosm approach using Lolium multiflorum. Oikos 104, 581–590.

Patamalai, B., Hill, D.W., Camp, B.J., Hejtmancik, E., Bridges, C.H., 1990. The isolation and identification of steroidal sapogenins in Kleingrass. Vet. Hum. Toxicol. 32, 314–318.

Pinares-PatiñO, C.S., Ulyatt, M.J., Waghorn, G.C., Lassey, K.R., Barry, T.N., Holmes, C.W., Johnson, D.E., 2003. Methane emission by alpaca and sheep fed on lucerne hay or grazed on pastures of perennial ryegrass/white clover or birdsfoot trefoil. J. Agric. Sci. 140, 215–226. https://doi.org/10.1017/S002185960300306X

Powell, J.M., Broderick, G.A., Grabber, J.H., Hymes-Fecht, U.C., 2009. Technical note: Effects of forage protein-binding polyphenols on chemistry of dairy excreta. J. Dairy Sci. 92, 1765–1769. https://doi.org/10.3168/jds.2008-1738

Probert, M.E., Delve, R.J., Kimani, S.K., Dimes, J.P., 2005. Modelling nitrogen mineralization from manures: Representing quality aspects by varying C/N ratio of sub-pools. Soil Biol Biochem. 37, 279-287.

Prosser, J.A., Speir, T.W., Stott, D.E., 2011. Soil Oxidoreductases and FDA Hydrolysis. In Methods of Soil Enzymology. R.P. Dick (editor) Soil Science Society of America Book series No. 9, Madison, USA.

Reed, J.D., 1986. Relationships among Soluble Phenolics, Insoluble Proanthocyanidins and Fiber in East African Browse Species. J. Range Manag. 39, 5. https://doi.org/10.2307/3899675

Robertson, G.P., Groffman, P.M., 2007. 13 – Nitrogen transformations. In: Paul, E.A., Soil Microbiology, Ecology and Biochemistry (Third Edition). Academic Press, San Diego, pp. 341–362.

Sinsabaugh, R.L., 2010. Phenol oxidase, peroxidase and organic matter dynamics of soil. Soil Biol. Biochem. 42, 391–404. https://doi.org/10.1016/j.soilbio.2009.10.014

Smith, J.L., McNeal, B.L., Cheng, H.H., 1985. Estimation of soil microbial biomass: an analysis of the respiratory response of soils. Soil Biol. Biochem. 17, 11–19.

Smolander, A., Kanerva, S., Adamczyk, B., Kitunen, V., 2012. Nitrogen transformations in boreal forest soils—does composition of plant secondary compounds give any explanations? Plant Soil 350, 1–26. https://doi.org/10.1007/s11104-011-0895-7.

Sparling, G.P., 1992. Ratio of microbial biomass carbon to soil organic carbon as a sensitive indicator of changes in soil organic matter. Aust. J. Soil Res. 30, 195-207.

Stewart, E., 2018. Environmental impacts from cattle consuming tannin-containing hays. MS Thesis, Utah State University, Logan, UT. In Press.

Subbarao, G.V., Rondon, M., Ito, O., Ishikawa, T., Rao, I.M., Nakahara, K., Lascano, C., Berry, W.L., 2007. Biological nitrification inhibition (BNI)—is it a widespread phenomenon? Plant Soil 294, 5–18. https://doi.org/10.1007/s11104-006-9159-3

Ta, T.C., Faris, M.A., 1987. Species variation in the fixation and transfer of nitrogen from legumes to associated grasses. Plant Soil. 98, 2, 265-274.

Tabatabai, M.A., 1994. Soil Enzymes, in: Methods of Soil Analysis: Part 2—Microbiological and Biochemical Properties, SSSA Book Series. Soil Science Society of America, Madison, WI, pp. 775–833.

Tava, A., Odoardi, M., Oleszek, W., 1999. Seasonal changes of saponin content in five alfalfa (Medicago sativa) cultivars. Agric. Mediterr. 129, 111–116.

Tilman, D., Isbell, F., Cowles, J.M., 2014. Biodiversity and Ecosystem Functioning. Annu. Rev. Ecol. Evol. Syst. 45, 471–493. https://doi.org/10.1146/annurev-ecolsys-120213-091917

Tilman, D., Snell-Rood, E.C., 2014. Ecology: Diversity breeds complementarity. Nature 515, 44–45.

Tracy, B.F., Foster, J.L., Butler, T.J., Islam, M.A., Toledo, D., Vendramini, J.M.B., 2018. Resilience in Forage and Grazinglands. Crop Sci. 58, 31-42. https://doi.org/10.2135/cropsci2017.05.0317

United States Department of Agriculture, Agricultural Research Service, Natural Resources Conservation Service, Soil Quality Institute. 2001. Soil Quality Test Kit Guide. Bulk Density Test. p 9. http://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_050956.pdf

Waghorn, G., 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production—Progress and challenges. Anim. Feed Sci. Technol. 147, 116–139. https://doi.org/10.1016/j.anifeedsci.2007.09.013

Waghorn, G.C., Shelton, I.D., McNabb, W.C., McCutcheon, S.N., 1994. Effects of condensed tannins in Lotus pedunculatus on its nutritive value for sheep. 2. Nitrogenous aspects. J. Agric. Sci. 123, 109–119. https://doi.org/10.1017/S0021859600067836

Wall, M.E., Eddy, C.R., McClennan, M.L., Klumpp, M.E., 1952. Detection and estimation of steroidal sapogenins in plant tissue. Anal. Chem. 24, 1337–1341.

Winder, R.S., Lamarche, J., Constabel, C.P., Hamelin, R.C., 2013. The effects of high-tannin leaf litter from transgenic poplars on microbial communities in microcosm soils. Front. Microbiol. 4. https://doi.org/10.3389/fmicb.2013.00290

Wolinska, A., Stepniewsk, Z., 2012. Dehydrogenase Activity in the Soil Environment, in: Canuto, R.A. (Ed.), Dehydrogenases. InTech. https://doi.org/10.5772/48294

Woodward, S.L., Waghorn, G.C., Laboyrie, P.G., 2004a. Condensed tannins in birdsfoot trefoil (Lotus corniculatus) reduce methane emissions from dairy cows, in: Proceedings of the New Zealand Society of Animal Production. New Zealand Society of Animal Production, Hamilton, 160–164.

Woodward, S.L., Waghorn, G.C., Lassey, K.R., Laboyrie, P.G., 2004b. Does feeding Sulla (*Hedysarum coronarium*) reduce methane emissions from dairy cows? In: Proceedings of the New Zealand Society of Animal Production, 62: 227-230.

Zak, D.R., Holmes, W.E., White, D.C., Peacock, A.D., Tilman, D., 2003. Plant diversity, soil microbial communities, and ecosystem function: are there any links? Ecol. 84, 8, 2042-2050.

Zakir, H.A.K.M., Subbarao, G.V., Pearse, S.J., Gopalakrishnan, S., Ito, O., Ishikawa, T., Kawano, N., Nakahara, K., Yoshihashi, T., Ono, H., Yoshida, M., 2008. Detection, isolation and characterization of a root-exuded compound, methyl 3-(4-hydroxyphenyl) propionate, responsible for biological nitrification inhibition by sorghum (Sorghum bicolor). New Phytol. 180, 442–451. https://doi.org/10.1111/j.1469-8137.2008.02576.x

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.

APPENDICES

APPENDIX A

SAPONIN ISOLATION AND CHEMICAL ANALYSIS

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 tephlon 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.

References:

Lee, S.T., Stegelmeier, B.L. Gardner, D.R., 2001. The Isolation and Identification of Steroidal Sapogenins in Switchgrass. J. Nat. Toxins. 10 (4): 273-281.

Patamalai, B., Hejtmancik, E., Bridges, C. H., Hill, D. W., Camp, B. J., 1990. The isolation and identification of steroidal sapogenins in kleingrass. Vet. Hum. Toxicol. 32(4), August, 314-318.

APPENDIX B

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Clemensen, A.K., Provenza, F.D., Lee, S.T., Gardner, D.R., Rottinghaus, G.E., Villalba, J.J., 2017. Plant Secondary Metabolites in Alfalfa, Birdsfoot Trefoil, Reed Canarygrass, and Tall Fescue Unaffected by Two Different Nitrogen Sources. Crop Sci. 57, 964-970. https://doi.org/10.2135/cropsci2016.08.0680

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I am requesting your permission to include the listed publication(s) in which you coauthored. I will include this letter in a special appendix of my dissertation.

Please indicate your approval of this request by signing in the space provided, attaching any other form or instruction necessary to confirm permission. If you have any questions, please call or email me.

Thank you for you cooperation,

Andrea Clemensen

I hereby give permission to Andrea Clemensen to reprint the following material in her dissertation:

Clemensen, A.K., Provenza, F.D., Lee, S.T., Gardner, D.R., Rottinghaus, G.E., Villalba, J.J., 2017. Plant Secondary Metabolites in Alfalfa, Birdsfoot Trefoil, Reed Canarygrass, and Tall Fescue Unaffected by Two Different Nitrogen Sources. Crop Sci. 57, 964-970. https://doi.org/10.2135/cropsci2016.08.0680

Signed: _____

March 16, 2018

Dear Dr. George Rottinghaus,

I am in the process of preparing my dissertation in the Wildland Resources Department at Utah State University. I plan to complete my degree program this semester.

I am requesting your permission to include the listed publication(s) in which you coauthored. I will include this letter in a special appendix of my dissertation.

Please indicate your approval of this request by signing in the space provided, attaching any other form or instruction necessary to confirm permission. If you have any questions, please call or email me.

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Clemensen, A.K., Rottinghaus, G.E., Lee, S.T., Provenza, F.D., Villalba, J.J., 2018. How planting configuration influences plant secondary metabolites and total N in tall fescue (Festuca arundinacea Schreb.), alfalfa (Medicago sativa L.) and birdsfoot trefoil (Lotus corniculatus L.): Implications for grazing management. Grass Forage Sci. 73, 94-100. https://doi.org/10.1111/gfs.12298

Clemensen, A.K., Provenza, F.D., Lee, S.T., Gardner, D.R., Rottinghaus, G.E., Villalba, J.J., 2017. Plant Secondary Metabolites in Alfalfa, Birdsfoot Trefoil, Reed Canarygrass, and Tall Fescue Unaffected by Two Different Nitrogen Sources. Crop Sci. 57, 964-970. https://doi.org/10.2135/cropsci2016.08.0680

Signed: _____

CURRICULUM VITAE

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EDUCATION

2007-2018 Ph.D. Candidate, Ecology-Wildland Resources, Quinney College of Natural Resources, Utah State University, Logan, Utah

2001-2006 B.S. Horticulture, College of Agriculture, Utah State University, Logan, Utah.

RESEARCH AND EXPERIENCE

Utah State University, 2007-present

Ph.D. Candidate

Advisors: Drs. Juan Villalba, Jennifer Reeve, Paul Grossl, Daniel Cook, and Tom Monaco.

Understanding Plant Secondary Metabolites, Above and Below Ground

My research evaluates how different management strategies and planting configurations might influence plant secondary metabolites in plants, as well as how plant secondary metabolites might influence soil dynamics in agroecosystems.

- 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.

TEACHING EXPERIENCE

Utah State University, 2009
Logan, UT
Teaching Assistant, FRWS 4900 - Managing Dynamic Ecological Systems

JOURNAL ARTICLES – PEER REVIEWED

Clemensen, A.K., G.E. Rottinghaus, S.T. Lee, F.D. Provenza, and J.J. Villalba. 2018. How planting configuration influences plant secondary metabolites in tall fescue (*Festuca arundinacea* Schreb.), alfalfa (*Medicago sativa* L.), and birdsfoot trefoil (*Lotus corniculatus* L.): implications for grazing management. Grass and Forage Science, 73, 94-100.

Clemensen, A.K., F.D. Provenza, S.T. Lee, D.R. Gardner, G.E. Rottinghaus, and J.J. Villalba. 2017. Plant Secondary Metabolites in Alfalfa, Birdsfoot Trefoil, Reed Canarygrass, and Tall Fescue Unaffected by Two Different Nitrogen Sources. Crop Science, 57, 964-970. https://doi.org/10.2135/cropsci2016.08.0680

Maughan, B., F. D. Provenza, R. Tansawat, C. Maughan, S. Martini, R. Ward, **A. Clemensen**, X. Song, D. Cornforth and J. J. Villalba. 2014. Importance of grass-legume choices on cattle grazing behavior, performance and meat characteristics. Journal of Animal Science, 92, 2309-2324.

Villalba, J.J., J. Miller, J.O. Hall, **A.K. Clemensen**, R. Stott, D. Snyder, and F.D. Provenza. 2013. Preference for tanniferous (Onobrychis viciifolia) and non-tanniferous (Astragalus cicer) forage plants by sheep in response to challenge infection with Haemonchus contortus. Small Ruminant Research, 112(1-3), 199-207. doi:10.1016/j.smallrumres.2012.11.033

Villalba, J.J., F. D. Provenza, **A.K. Clemensen**, R. Larsen, and J. Juhnke. 2011. Preference for diverse pastures by sheep in response to intraruminal administrations of tannins, saponins, and alkaloids. Grass and Forage Science, 66(2): 224–236.

Provenza, F.D., J.J. Villalba, R.W. Wiedmeier, T. Lyman, J. Owens, L. Lisonbee, **A.K. Clemensen**, K.D. Welch, D.R. Gardner and S.T. Lee. 2009. Value of Plant Diversity for Diet Mixing and Sequencing in Herbivores. Rangelands: February 31(1): 45-49.

BOOK CHAPTERS – PEER REVIEWED

Clemensen, A.K. and F.D. Provenza. 2010. Environmental Effects on Concentrations of Plant Secondary Compounds: Finding a Healthy Balance. In Panter K.E., Wierenga T.L., and Pfister J.A. Poisonous Plants: Global Research and

Solutions. CABI Publishing, Cambridge, MA.

POSTERS

Clemensen, A., T. Lyman and B. Burritt. 2008. Dust-to-Dust: Dynamism between soils, plants, herbivores and people. 2008 BEHAVE Meeting. October 28. Park City, UT.

INVITED PRESENTATIONS

Clemensen, A.K. 2008. Linking the Health of Soils and Plants. 2008 BEHAVE Meeting. October 28-30. Park City, Utah.

OTHER PRESENTATIONS

Clemensen, A.K, J. Reeve, and J. Villalba. 2014. Understanding Plant Secondary Compounds in Grazing Systems; Above and Below Ground. Society for Range Management 67th Annual Meeting. Orlando, Florida.

Clemensen, A.K. 2009. Environmental Effects on Concentrations of Plant Secondary Compounds: Finding a Healthy Balance. Eighth International Symposium on Poisonous Plants. João Pessoa, Paraíba, Brazil.

Clemensen, A.K. 2009. Environmental Effects on Concentrations of Plant Secondary Compounds: Finding a Healthy Balance. Society for Range Management 62nd Annual Meeting. Albuquerque, New Mexico.