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ENHANCING THE PRODUCTION AND SUSTAINABILITY OF PASTURE-FED

BEEF USING NON-TRADITIONAL LEGUME FORAGES

by

Andrea I. Bolletta

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

of

DOCTOR OF PHILOSOPHY

in

Plant Science

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UTAH STATE UNIVERSITY
Logan, Utah

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ABSTRACT

Enhancing the production and sustainability of pasture-fed beef using non-traditional

legume forages

by

Andrea I. Bolletta, Doctor of Philosophy

Utah State University, 2020

Major Professor: Dr. Jennifer W. MacAdam
Department: Plants, Soils and Climate

Conventional beef production systems, which include intensively managed

feedlots, are of concern to the public due to diverson of cereal grains to high-grain cattle

diets and the environmental impacts associated with ruminant production. These include

contamination of groundwater and water bodies with antibiotics and hormones, and

emissions of greenhouse gases (GHG) such as enteric methane (CH_4), nitrous oxide

(N_2O) and CO_2. Some alternative beef production systems have been proposed, like grass

fed-beef, but still present some disadvantages such as lower feed efficiency conversion,

requiring longer finishing periods, and greater numbers of animals and land to produce

the same quantity of beef product as feedlot systems, and greater CH_4 emissions due to

lower quality of the diet. Therefore, mitigation strategies are needed to counter these

negative impacts and support healthy soils, flora, fauna, and water resources that, in turn,

can sustain natural ecological processes (e.g., the nutrient cycle, water cycle, and energy

flow). Mitigation can be addressed through the use of alternative legume species such as

tannin-containing birdsfoot trefoil (BFT) and non-tannin containing cicer milkvetch

(CMV) for beef finishing. Tannin-containing temperate perennial legumes are bloat-safe, fix their own nitrogen (N) and provide greater nutritional valuable similar to concentrates when grown under irrigation in the Mountain West. This study demonstrated that legume-based pasture finishing can be used to reduce GHG emissions and enhance N and carbon (C) utilization for both tannin-containing and non-tannin temperate legumes. In a field study, legume forage quality resulted in greater dry matter intake per unit of respired enteric CH_4 than for the grass, resulting in a reduced C footprint for beef production on legume pastures. This study demonstrated greater soil C sequestration under meadow bromegrass (MB) and small burnet (SB), a hydrolysable tannin-containing forb, mainly in the uppermost (0-10 cm) soil layer where greater soil microbial activity responded to greater soil warmth and oxygenation, and greater turnover of fine roots and root C exudation. Therefore, adoption of well-adapted perennial legumes for beef production can reduce the negative environmental impacts associated with traditional forage-based beef production systems, while improving the profitability of beef production and reducing the time spent grazing through more rapid rates of gain. Even greater soil C sequestration can be achieved with highly productive grasses and forbs.

In an *in vitro* study, legume hay digestibility ranged between 69 and 77%. Of these legumes, cicer milkvetch demonstrated greater *in vitro* dry and organic matter digestibility than alfalfa, BFT, and sainfoin as well as MB and small burnet, likely due to its greater leaf proportion and vine-like stems with less structural tissue. Residual tannins in fiber did not impede microbial fermentation but may have impacted the rate of rumen microbial colonization. Greater time to reach half cumulative gas production of MB

during *in vitro* fermentation likely could be explained by slower rumen microbial colonization due to physical constraints, given the longer, larger fiber bundles in grasses. Greatest dry matter intake would be expected for the legumes, due to their higher fermentation rates at the beginning of the incubation process and shorter half-time to maximum asymptotic cumulative gas production, resulting in lower total gas production for all legumes, faster rates of passage and reduced rumen fill.

In a controlled environment study, greater organic N and C concentrations in the uppermost soil layer were likely due to greater root proliferation resulting from manure deposition. Total soil N ha^{-1} was greater for MB and SB than for BFT and CMV and total soil organic C was greatest for BFT and least for MB, suggesting a role for tannin in reducing N mineralization and nitrification rates, and preventing N losses through nitrate leaching, ammonia volatilization and N_2O emissions from the pastures systems. Greater root mass accompanied by greater total root C and N in MB columns did not convert on greater soil C storage. When N balances were developed for four simulated grazing systems of BFT, CMV, MB and small burnet, SB and MB gained significant soil organic N, thereby enhancing soil quality and carbon sequestration. These results ultimately have the potential to alleviate a number of the concerns associated with ruminant production systems and improve ecosystems services.

(253 pages)

PUBLIC ABSTRACT

Enhancing the production and sustainability of pasture-fed beef using non-traditional
legume forages

Andrea I. Bolletta

Despite the increasing worldwide demand for beef as a protein source, consumers
are concerned about the sustainability of ruminant production systems. Their main
concerns are animal welfare for feedlot-fed animals, greenhouse gas (GHG) emissions,
global warming and worker safety. Traditional feedlot-based beef production systems
have been associated with locally greater levels of soil, water and air contamination, as
well as the overuse of antibiotics and growth hormones. The use of legume pastures such
as cicer milkvetch (CMV) and birdsfoot trefoil (BFT), which fix their own nitrogen (N)
and often contain beneficial secondary compounds such as tannins and provide for rapid
gain and improved meat quality, holds promise as an alternative strategy to feedlots for
beef finishing. These legumes can mitigate GHG emissions without reducing beef
productivity and improve enterprise profitability when sold locally as natural or organic
pasture-finished meat. Tannins can be beneficial to ruminants or some types, especially in
high concentrations, can have anti-herbivore properties. The condensed tannins
synthesized by BFT are known to prevent bloat and to enhance the production of
ruminants. More generally, tannins are beneficial not only to the plants that accumulate
them, but can also slow soil mineralization of organic matter, better matching N release
to plant uptake. Ruminants can convert fibrous feedstuffs not suitable for human
consumption, such as corn stalks, into sources of high-quality protein for human

consumption, and thrive without grain on pastures and hay produced on marginal land that is not suitable for cultivation. Legumes pay a key role in the mitigation of environmental impacts of beef production, because their elevated forage quality increases digestion rate, intake and animal gain, their tannins improve the efficiency of rumen N utilization, and their quality and tannin concentrations both tend to reduce enteric CH_4 emissions and N losses. Likewise, plant litter and manure from tannin-containing species would help to sequester N and carbon in the production system, helping to achieve sustainable beef production. Evaluation of the sustainability of ruminant production systems should be based on their environmental impact, the nutritive value of the food produced, the appropriate use of agricultural land, and the economic sustainability of producers and their rural communities.

To my loving family

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1 INTRODUCTION

Over the next 40 years, consumption of animal protein is expected to increase by more than 60% as a result of increased demand for meat inclusion in human diets from developing countries, primary from Asia and Africa (FAO, 2009; Smith et al., 2018). In developed countries where meat consumption is already high, there is growing interest in livestock produced in sustainable systems with enhanced ecosystem services, including biodiversity, enhanced soil quality, clean air and water, animal health and welfare, quality and safety of meat products, and fair wages and safe working conditions for agricultural workers as well (Gerber et al., 2013; Hristov et al., 2013). At the same time, sustainability of beef production has been criticized in the context of global food security and environmental issues because of the relatively high land use, the use of agrochemicals (pesticides, herbicides, fertilizers), the use of pharmaceuticals and other substances in animals (vaccines, antibiotics, medicated feeds, growth hormones), a low feed conversion efficiency in grain-fed ruminants, and the high greenhouse gas (GHG) emissions per kg of meat produced compared with poultry and swine (Bouwman et al., 2013; Ripple et al., 2014). In this sense, the livestock sector is responsible for 14.5% of anthropogenic global GHG emissions (FAO, 2019) and 2.1% of the total US anthropogenic GHG emissions (US EPA, 2019).

In the United States, conventional beef cattle systems are based on cereal grain and require annual nitrogen (N) fertilization and periodic replacement of the soil phosphorus (P) and other mineral nutrients which are removed with harvested grain. The routine use of chemical fertilizers, insecticides and herbicides may degrade soil chemistry, decreasing overall soil quality over time (National Research Council, 2010). Overuse of such inputs can result in water and air pollution, reductions in soil organic matter and soil pH (Geisseler and Scow, 2014), and increases in nitrate (NO_3^-) leaching and reactive N gas production (Robertson and Vitousek, 2009; Fowler et al., 2013). The production of annual grain crops requires mechanization and fuel for cultivation, planting and harvesting, which are also associated with significant nutrient loss to the environment and soil loss through degradation of soil structure that leads to lower water infiltration and reduced nutrient holding capacity. Such agricultural practices contribute to NO_3^- leaching into potable water, eutrophication and the release of N oxides into the atmosphere, with both economic and environmental significance. The most damaging greenhouse gas from agriculture is nitrous oxide (N_2O) from management activities such as fertilizer use, manure application, and the utilization of N-fixing crops. These losses have been linked to human health issues (Vitousek et al., 1997), either directly as in the case of particulates created when NO_3^- and NH_4^+ combine, or indirectly when N_2O causes ozone depletion, thereby increasing UV-B radiation.

Another significant contributor to GHG emissions associated with ruminant agriculture is methane (CH_4) which is released by cattle and sheep during enteric fermentation and is a by-product of some approaches to manure management. The

agricultural sector is the largest contributor to N_2O and CH_4 emissions in the United

States; both have higher global warming potential than carbon dioxide (CO_2). Therefore,

is crucial to better understand production systems with respect to such N and C losses,

which are elevated relative to emissions in the feedlot phase of beef production due to the

degradation of excess protein in the rumen and the slow fermentation of high-fiber feeds

in the rumen, resulting in lower DM intakes. A lower proportion of the ingested N from

grazed herbage is retained than that in total mixed ration diets because, at least in

irrigated or well-watered forages, the protein concentration is not well-matched by

readily accessible carbohydrates (Haynes and Williams, 1993). Furthermore,

conventional ruminant milk production and feedlot finishing diets makes use of

antibiotics, medicated feeds, and growth hormones which allow animals to have

enhanced growth and production and reduced time to slaughter (Capper, 2012). But these

antibiotics and hormones from animal waste infiltrate the soil and can contaminate

surface and groundwater (Kemper et al., 2008; Arikan et al., 2009); the situation is

compounded when manure containing these substances is used as fertilizer.

In western North American beef systems, 80% of GHG emissions occur in the

cow-calf phase (Beauchemin et al., 2011). This includes emissions from cattle and their

manure, as well as indirect emissions from the production of feed and manufactured

inputs such as fertilizer and herbicides (Beauchemin et al., 2010). On a CO_2-equivalent

basis, 63% of these GHG emissions were due to enteric CH_4, and 84% of these enteric

CH_4 emissions were due to mother cows (Beauchemin et al., 2010). Methane is globally

important because it has significant potential as a GHG, it controls the oxidizing potential

in the remote atmosphere, affects stratospheric ozone by contributing water vapor, and its concentration has been rising rapidly (Munger, 2004). The use of alternative perennial legumes in pastures and as hay is a mitigation strategy that can curb many of the negative environmental impacts of beef production. For instance, perennial forages are productive for multiple years after establishment without additional cultivation or planting, eliminating soil and sequestered carbon losses associated with cultivated soils. Further, pasture legume species such as birdsfoot trefoil (*Lotus corniculatus* L.; BFT) and cicer milkvetch (*Astragalus cicer* L.; CMV) are non-bloating and have greater nutritive value than forage grasses such as meadow bromegrass (*Bromus biebersteinii* Roem. & Schult.; MB), because they contain less fiber, have greater fiber digestibility and more protein and non-fibrous carbohydrates (NFC) than grasses (Waghorn and Clark, 2004), providing a protein-to-carbohydrate ratio that better matches a ruminant's nutritional requirements (Brummer et al., 2016), improving cattle digestion and intake. Enteric CH_4 emissions would be expected to be reduced (Hart et al., 2009) due to improvements in forage quality and digestibility, which would enhance dry matter intake, and increase the ruminant production of propionate relative to acetate, reducing CH_4 production and enhancing ruminant performance.

Legumes also fix N by converting dinitrogen (N_2) gas into the amino group (-NH_2), supporting protein synthesis and increasing the quality and amount of herbage biomass in the pasture. To the extent chemical N fertilization is replaced with N_2 fixation and used in support of plant growth, N_2O emissions can be curtailed. Including non-leguminous forbs such as small burnet (*Sanguisorba minor* Scop.; SB) in pastures can

increase rooting diversity, benefit soil microbial ecology, and provide a range of secondary compounds with potential benefit to ruminants that are not found in grasses. Plant primary metabolites are associated with the growth and development of plants and are the products or substrates of essential metabolic processes such as photosynthesis and respiration. Plants also produce metabolites that are not directly involved in growth and development, known as secondary metabolites, which provide numerous benefits to the plant by attracting pollinators and seed dispersers, helping plants recover from injury, protecting plants from ultraviolet radiation, and aiding in defense against abiotic stresses, pathogens, diseases, and herbivores. Additionally, secondary metabolites may also provide benefits to ruminants and soils.

Greater forage quality coupled with plant secondary compounds like condensed tannins (CT) in BFT may improve nutrient utilization by ruminants or reduce CH_4 emissions (Jayanegara et al., 2009), and hydrolysable tannins (HT) such as those found in SB have been found to reduce N excretion from animals (Stewart et al., 2019) or in limited concentrations and in combination with CT, to increase ruminant growth rate compared with grasses (Aguerre et al., 2016), representing a sustainable means of reducing environmental impacts of ruminants. Further, tannins in plant litter and manure from animals consuming tannin-containing species has been found to reduce N mobilization in soils, reduce nutrient leaching, and increase N and C storage (Bradley et al., 2000; Smolander et al., 2012), contributing to soil health. Non-tanniferous forages such as grasses in properly managed perennial pastures have been found to increase the rate of C sequestration by 20% (Guo and Gifford, 2002; Conant et al., 2003) through the

incorporation of plant residues and manure, thus helping to improve the soil quality and functionality (Edmeades, 2003; Diacono and Montemurro, 2010).

The more quickly that weaned ruminants gain body weight, the lower the rate of GHG emissions of a beef production system (Peters et al., 2010). Sheep fed BFT had greater absorption of essential amino acids from the intestines than sheep fed big trefoil (*Lotus pedunculatus* Cav.), but no decrease in the synthesis of microbial protein in the rumen (Min et al., 2003). The CT synthesized by big trefoil has twice the molecular weight of BFT CT (Mueller-Harvey, 2006) and may be less able to release protein at the reduced pH of the abomasum than BFT CT. Lambs fed BFT gained more weight than lambs fed alfalfa (*Medicago sativa* L.) (Douglas et al., 1995), and much of the difference was shown to be due to the tannin in BFT (Douglas et al., 1999). The improved gains or milk production of cattle grazing or fed hay of BFT has been associated with greater NFC contents (MacAdam and Griggs, 2013; MacAdam and Villalba, 2015) and the presence of CT in BFT (Waghorn, 2008; Wang et al., 2015; Villalba et al., 2019) as well as its greater fiber digestibility (Christensen et al., 2013; Hunt et al., 2014). Birdsfoot trefoil condensed tannins have been shown to improve meat production by increasing plant protein utilization, due to tannins bind to proteins in the rumen which are later released in the abomasum (Barry and McNabb, 1999; Mueller-Harvey, 2006; Waghorn, 2008). In addition to these nutritional benefits, CT in some forage legumes prevent bloat and help control internal parasites and nematodes (Hoste et al., 2006). Improved gains reduce the time needed to reach slaughter weight which also reduces manure added to the system, which is the primary source of N and P pollution from beef systems (Gurian-Sherman,

2011). The meat of pasture-finished beef has the additional benefit of lower saturated fat content and a higher omega-3 fatty acids, both of which are considered beneficial to human health, which appeals to consumers (Daley, 2010; Chail et al., 2016).

Finally, consumption of forages reduces competition for grain with humans (in a world with an increasing population) and livestock such as swine and poultry. Beef cattle can be raised without grain on agricultural land that should not be cultivated because of slope, soil depth, or other physical or climatic limitations. Well-managed perennial forage crops and grazing lands contribute many ecosystem services such as C sequestration and filtration of nutrient-rich runoff. These benefits along with nutrient cycling and mitigation of climate change should be accounted for in assessments of beef production (Teague et al., 2016). Biologically fixed N reduces both input costs and N losses to the environment (Muir et al., 2014). Thus, beef production systems under alternative legumes such as BFT and CMV not only improve animal performance and welfare but also reduce the C and N footprint and reduce inputs and more rapid cattle gains improve the profitability for farmers, especially in the case of local marketing.

2 LITERATURE REVIEW

2.1 Ruminant greenhouse gas emissions

Ruminant production is the focus of public scrutiny because cattle and other ruminants are the major source of GHG emissions from agricultural livestock production systems (Ripple et al., 2014). These GHG emissions include CH_4, N_2O, and CO_2 (Rotz et al., 2019). Methane is a GHG 28 times more potent than CO_2 (IPCC, 2013), primarily

produced in the rumen and exhaled through the mouth and nose as a normal by product of

digestion (Murray et al., 1976); representing wasted food energy that ranges between 2 to

12% of the gross energy consumed with the diet (Johnson and Johnson, 1995) and

resulting in a negative environmental impact. The reduction of CO_2 to CH_4 by

methanogenic archaea acts as a hydrogen gas (H_2) sink, removing H_2 from the rumen and

avoiding the negative effects of H_2 accumulation on microbial enzymatic activity and

degradation of plant material (McAllister and Newbold, 2008). Methanogens use H_2 as

their main energy source, producing CH_4 in the process through the following reaction:

$$CO_2 + 4\ H_2 = CH_4 + 2\ H_2O$$
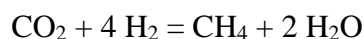
The enteric CH_4 emissions of grazing cattle can be measured using the sulfur

hexafluoride (SF_6) tracer gas technique, which allows direct measurement of individual

grazing animals without interrupting grazing (Johnson et al., 1994; Johnson et al., 2007).

According to Johnson and Ward (1996), the loss of enteric CH_4 to the atmosphere varies

by ruminant species, geographical location, feed quality, feed intake, feed composition,

and the processing of the feed. Indeed, the most meaningful basis on which to report

enteric CH_4 emissons is as a function of dry matter intake. Forages with greater fiber

concentration have increased ruminal retention time that constrains rate of passage

(Allen, 1996; Meyer et al., 2010). If feed retention time in the rumen is increased, CH_4

production per unit of forage intake is expected to increase, since the extent of rumen

fermentation is increased and there is more H_2 to be used as a substrate for methanogenic

bacteria (Moss et al., 2000). In addition to this, a more fibrous diet usually results in a

greater ratio of acetate to propionate, which is correlated with increased CH_4 production (Johnson and Johnson, 1995; Ominski and Wittenberg, 2004).

When grain prices are subsidized, finishing livestock on cereal diets is profitable, but the environmental cost of soil erosion and the resulting movement of nutrients into lakes and other outlets is not factored into the cost of grain finishing. The incorporation of highly digestible legume forages is a sustainable economic alternative to grain finishing, since input costs are minimal, and it is environmentally sustainable because enteric CH_4 emissions are also reduced compared with other forages due to the impact on ruminant microbes and volatile fatty acid (VFA) production (Johnson et al., 2007). Pasture legumes species such as birdsfoot trefoil (*Lotus corniculatus* L.; BFT) and cicer milkvetch (*Astragalus cicer* L.; CMV) are non-bloating and have greater nutritive value than forage grasses such as meadow bromegrass (*Bromus biebersteinii* Roem. & Schult.; MB), due to their lower fiber, greater fiber digestibility and greater nonfibrous carbohydrates (NFC) and crude protein (MacAdam and Griggs, 2013; MacAdam and Villalba, 2015). They are rapidly fermented in the rumen, even as mature forages (Phelan et al., 2015). These characteristics result in lower retention times in the rumen, so intake and production are higher than those for forage grasses (Van Soest, 2018). This faster rate of forage legume digestion is primarily attributed to the faster rates of particle breakdown and faster fermentation in the rumen (Waghorn et al., 1989).

Non-fibrous carbohydrates such as starch, fructans, and cell wall components such as pectin are a readily fermentable sources of energy for microorganisms in the rumen, providing energy in synchrony with the high crude protein concentrations of

forage legumes for the synthesis of microbial protein (Berthiaume et al., 2010). In addition, forage legumes do not decline in N concentration (Pelletier et al., 2010) and digestibility (Dewhurst et al., 2009) at the same rate as grass forages with progressing maturity. The higher nutritional composition of legumes usually leads to greater DM intake in ruminants than that observed for grasses (Phelan et al., 2015) resulting in greater average daily gain (MacAdam et al., 2011; MacAdam and Villalba, 2015; Pitcher, 2015) which decreases substantially the days to slaughter and the amount of GHGs emitted (specially CH_4) per unit of intake or red meat relative to cattle fed grasses (Phelan et al., 2015). Perennial legume forages yield ruminal microorganism proportions similar to those of grain-fed ruminants, increasing proportions of propionate-forming bacteria and decreasing H_2 production and resulting in decreased CH_4 emissions relative to forages with a lower content of non-fibrous carbohydrates (Sun et al., 2015), curbing many of the negative environmental impacts involved in the beef production. Decreasing enteric CH_4 emissions from ruminants while improving ruminant production is desirable both as a strategy to reduce global agricultural GHG emissions and as a means of improving feed conversion efficiency (Martin et al., 2010).

The three plant secondary compounds (PSC) most effective in reducing CH_4 emissions *in vitro* are tannins, saponins, and essential oils, because all of these compounds are toxic to protozoa, and rumen methanogens associate with rumen protozoa that generate H_2 (Martin et al., 2010). The tannins produced by each plant species is unique to that species, and the impact is related to the concentration of these PSC in plant tissues and on the biological activity of the tannin, dictated by its subunit composition

(Schofield et al., 2001). According to Waghorn (2017), the concentration of condensed tannins (CT) is less important than its structure in reducing enteric CH_4 emissions. However, the mechanism by which CT reduces CH_4 emissions is not entirely clear. Some researchers believe that CT indirectly affects the production of hydrogen ions or that through they have an antibiotic effect on rumen microflora involved in CH_4 production (Tavendale et al., 2005). In BFT, tannins are present in low concentration and do not constrain animal intake (Ramirez-Restrepo et al., 2016). Tannins bind and precipitate proteins, thereby reducing the incidence of bloat in ruminants and protecting dietary proteins in the rumen from microbial digestion (Min et al., 2003). The BFT tannin-protein bond that forms at the near-neutral pH of the rumen is reversible at the lower pH of the abomasum, where gastric digeston occurs (Waghorn, 2008). Reduced proteolysis in the rumen reduces the formation of NH_3 and excretion of N in urine or milk. The CT produced by BFT binds excess plant proteins in the rumen but allows this protein to be released in the abomasum where it can be digested and amino acids absorbed from the intestines (Waghorn et al., 1987; Mueller-Harvey, 2006; Waghorn, 2008). Consequently, ruminants grazing BFT have enhanced nutrition and performance relative to ruminants grazing other perennial forages, since more N is retained in the animal's tissues (Carulla et al., 2005; Stewart et al., 2019). Ultimately, these benefits appear as enhanced meat (Wen et al., 2002; MacAdam et al., 2011) and milk (Woodward et al. 2004; Turner et al., 2005) production. Greater rates of gain or milk production result in greater agricultural sustainability (Ramirez-Restrepo and Barry, 2005).

2.2 Fiber digestibility and intake

Grain is fed to ruminants in total mixed (balanced) rations because the starch available in grain provides excess energy that contributes to rapid weight gains or abundant milk production. However, ruminants do not require concentrates such as grain because they can derive the energy they need from the cellulose in forages and feeding high-starch grains that lower rumen pH opens ruminants to infection and necessitates prophylactic antibiotic use. The ability of ruminants to use plant fiber for energy places ruminants in a unique position in the world's economy (Van Soest, 2018), an advantage that is lost when concentrates are used as ruminant feed. Therefore, rather than feeding high-starch grains that that are inefficiently converted to meat during the finishing phase, cattle produced in the Mountain West can be finished on perennial legumes forages that have been found to accumulate assessible (non-fibrous) carbohydrates to levels comparable to a concentrate ration (Chail, et al., 2016). These non-fibrous carbohydrates are readily used for energy and are well-matched with the high crude protein concentration of legumes, particularly when bloat is controlled by tannins.

In ruminant digestion, intake rate is controlled by the digestibility of the diet which is a function of the fiber digestibility and concentration, because indigestible fiber will limit the rate of passage of forage from the rumen and thus limit intake. *In vitro* rumen fermentation (Theodorou et al., 1994) can be used to compare the rate and extent of digestion of different ruminant feed sources. The influence of fiber, particularly its lignin concentration, and tannins on forage digestion and fermentation dynamics is not

well-understood for Mountain West-grown perennial forage legumes with uniquely elevated non-fibrous carbohydrates.

In general, lignin is a cell-wall component that is effectively indigestible by rumen microbes, and thereby interferes with fiber digestion, limiting forage intake. Legumes and grasses differ in the concentration, composition and physical location of this component in their tissues (Jung, 1989; Hoffman et al., 1993; Wilson, 1993). While legumes contain more lignin than grasses, the lignin present in grasses strongly inhibits cell wall digestibility due to an alternative chemical composition (Jung, 1989), with an overall negative impact on animal performance. Lignin only accumulates in the walls of xylem (water-carrying) and fiber (structural) cells. These cells have thick secondary walls, and lignin is most concentrated at their perimeter. Therefore, there is a physical constraint in the microbial digestion of fiber walls, wherein digestion occurs most readily closest to the center of the cell, from its lumen (Wilson and Mertens, 1995). The veins in grasses, where bundles of fiber cells occur, can run from the base of grass sheaths to the tips of leaves, while the vein structure in legume leaves is reticulate, or net-like, with short runs of fiber cells. This difference in forage plant morphology is evidenced by greater concentrations of neutral detergent fiber (NDF) in grasses than in legumes. The concentration of lignin in legumes is generally greater than in grasses because a given legume fiber cell will have a greater concentration of lignin than a grass fiber cell, but the greater fiber concentration of grasses results in a greater impediment to digestion and intake. Legumes also retain greater nutritive value (crude protein and non-fibrous carbohydrates along with lower contents of fiber) as they mature compared with grasses

(Waghorn and Clark, 2004; Brummer et al., 2016), maintaining a greater rate of forage digestion, leading to greater intake. However, there are also significant differences among legumes; for example, BFT and CMV have greater average concentrations of *in vitro* digestible dry matter than alfalfa and sainfoin because of greater stem digestibility (BFT) or leaf proportion (CMV) (McGraw and Marten, 1986) that improve voluntary intake and ruminant production.

The condensed tannins synthesized by BFT and sainfoin can have positive effects on ruminant health and nutrition, but do not have a negative impact on DM intake or fiber digestion (Ramírez-Restrepo and Barry, 2005; Ramírez-Restrepo et al., 2006). In fact, tannins precipitate excess protein in the rumen, allowing then to be released in the abomasum (Waghorn et al., 1987) where impact on greater animal performance and lower emissions of NH_3 to the environment. Several studies have reported that CT concentrations must be greater than 5% to reduce fiber digestion through formation of a CT-microbial enzyme complex (Barry and Manley, 1986; Bae et al., 1993; Min et al., 2003) that can inactivate microbial enzymes that digest rumen contents (Reed, 1995). Incorporating alternative tannin-containing forages into ruminant diets has the potential to benefit ruminants as well as the environment.

2.3 Nitrogen cycling

The specific chemical composition of a plant, and the amount of litter and root turnover it creates every year, affects soil organic matter concentrations as well as the physical and chemical properties of the soil. Indeed, soil structure is considered an

indicator of soil health because good soil structure allows adequate water infiltration rates, improves water holding capacity, and contributes to maintaining porosity for gas exchange. These factors influence the rooting depth of plants and the habitat for soil microbes (Horwath, 2007). In turn, soil organisms are responsible for the life cycle of the soil ecosystem through decomposition of litter and roots, nutrient recycling, creating cation exchange capacity and nutrient retention. An optimal balance of C and N in the soil is critical for maintaining soil organisms. The decomposition of plant residue and organic material, microbial N-fixation, and nutrient mineralization are important processes in the soil that sustain agricultural ecosystems. The uppermost layers of the soil that are well-oxygenated, warmer, and that receive fecal and urine waste, are therefore where most soil microbes reside and maintain high levels of activity. The majority of plant root growth and nutrient recycling also occurs in the upper soil layers, so understanding soil dynamics improves our understanding of plant growth and development.

Nitrogen is the nutrient most commonly limiting for plant growth. Nitrogen comprises about 79% of atmospheric gases, and 99% of atmospheric N is in the form of N_2, which is inert and cannot be used by most living organisms. However, biological nitrogen fixation (BNF) is a natural process by which certain prokaryotic microorganisms fix this atmospheric N using a highly specialized enzyme complex, nitrogenase. In this process, a molecule of N_2 is reduced to two molecules of NH_3 and immediately used to form organic compounds that can be metabolized to amino acids within the plant. In fertilized crops, excess soil NO_3^- is an environmental concern because it is not readily

adsorbed to soil mineral particles and organic matter and can be leached into ground water by excessive irrigation or precipitation (Pierzynski et al., 2000). Thus, BNF in association with legumes is an environmentally benign and sustainable alternative to chemical N fertilization.

The mineralization of organic matter results in the formation of NH_4^+, which then is rapidly converted to NO_3^- by the process of nitrification, and NO_3^- may accumulate in the soil solution to high concentrations (Norton, 2008). Nitrate is more mobile than NH_4^+ due to its negative charge, and it is easily lost through leaching and denitrification (Prosser, 1989; Norton, 2008). Consequently, N mineralization and nitrification are key N transformations that largely determine the availability and mobility N in soils, mediating plant N uptake, NO_3^- leaching and N_2O gas emissions (Norton, 2008; Norton and Stark, 2011). Living cells can use N as either NO_3^- or NH_4^+, but if it is available, the assimilation of NH_4^+-N costs plants and microbes less metabolic energy than the assimilation and reduction of NO_3^--N (Schlesinger, 1997). Another key component in the agricultural N cycle is that N_2O is a potent GHG, with 265-298 times greater global warming potential than CO_2 for absorbing energy, warming the earth and slowing the rate at which heat escapes to space (IPCC, 2013). This gas is produced in soils by microbial nitrification (the oxidation of NH_4^+ to NO_3^-) and denitrification (the reduction of NO_3^- to N_2) (Stevens et al., 1997) as an intermediate of each process. Soil N sources that can result in N_2O production and emission include mineral fertilizer, manure, crop residues (legume crop residues usually decompose faster than residues from non-legume crops), and BNF of atmospheric N_2 by legume crops (Rochette and Janzen, 2005; Schmeer et al.,

2014), which include both annual legumes such as soybeans and perennial forage legumes. In legumes systems, N_2O can also be emitted from the degradation of root nodules, although these nodules are not a storage site for N. The organic N in nodules comprises the enzymes needed for respiration and N fixation as well as the leghemoglobin pigment employed to carry oxygen to mitochondria. Nodule N is mineralized to NH_4^+, followed by nitrification and denitrification that produce N_2O (Itakura et al., 2013). The magnitude of N_2O emissions depends on soil conditions, including the oxygen and soil water content, concentrations of NH_4^+ and NO_3^-, soil temperature, and other climatic conditions (Rochette et al., 2004). Soil compaction and irrigation both decrease soil oxygen and therefore increase the emission of N_2O (van Groenigen et al., 2005).

Grazing affects ecosystem structure and function, both above and below ground, since animals grazing on a pasture add manure, which recycles plant nutrients back into the soil; indeed, 60-90% of the ingested nutrients are recycled, increasing soil microbial C and N (Wang et al., 2006). Poorly timed grazing, following irrigation or precipitation, can compact soil and damage soil structure. Previous studies (Jarvis et al., 1989; Jarvis et al., 1996; Delve et al, 2001) have reported that urine N deposition is directly related to the N concentration of the diet. Under grazing, most of the N in urine from cattle is in the form of urea, which is rapidly converted to plant-available N, some of which is immediately lost as NH_3 gas (Whitehead, 1995). The N deposited as urine is converted to NO_3^- and becomes susceptible to losses through N leaching or N gas emissions (Getachew et al., 2006), and ultimately, may contribute to eutrophication in bodies of

water such as Utah Lake or Mantua Reservoir (Zonderland-Thomassen et al., 2014; Leip et al., 2015), or to the pollution of air and drinking water. Feces contain organic N that is less available than the N in urine because it must be mineralized by soil microbes to become plant-available N (Haynes and Williams, 1993; Menneer et al., 2004). Clearly, reducing in the proportion of N partitioned into urine and increasing N retention in ruminants or at least increasing the partitioning of N into feces, will be beneficial for the environment, since urinary N is much more susceptible to gaseous and leaching losses than fecal N (Cai et al., 2017).

The incorporation of forages with plant secondary metabolites such as CT and HT into plant litter and manure can play a key role regulating soil N cycling in beef production systems. Plant secondary metabolites affect plant and fecal decomposition rates, the activity of soil microflora and fauna and their enzyme activity, as well as C and N sequestration (Bradley et al., 2000; Smolander et al., 2012; Adamczyk et al., 2013). An excess of soil carbon can inhibit N mineralization by increasing N immobilization, but an optimal balance of soil N and C supports the sequestration of soil C and organic N, reducing nutrient loss to the environment.

A moderate concentration of tannins from a few plant species (birdsfoot trefoil, sainfoin and sulla) in ruminant diets is known to have beneficial effects on ruminants (Waghorn, 2008). For instance, in a study of dairy cows fed perennial ryegrass with increasing proportions of added BFT, N excretion was decreased in urine and increased in feces (Woodward et al., 2009). Misselbrook et al. (2005) found that feeding BFT in place of alfalfa in total mixed rations reduced NH_3 emissions from manure by 25 to 45%,

due to improved protein use and reduced urea excretion by 55% (Lagrange et al., 2020).

These effects were considered to be due to BFT CT in both studies through the protection

of dietary protein by CT in the rumen and release of protein in the abomasum and its

absorption in the intestines. Some tannins, such as the HT in SB, bind to protein

irreversibly, increasing the excretion of N in feces and reducing excretion in urine, but

depriving the ruminant of dietary crude protein (Stewart et al., 2019). The incorporation

of perennial tannin-containing legumes, which are productive for multiple years, not only

would contribute to an increase in soil OM (C sequestration) improving soil health and

ultimately increasing the yield and quality of the pasture, while eliminating the need for

external N input as chemical fertilizer.

3 OBJECTIVES AND HYPOTHESES

The main objective of this study was to examine how the incorporation of

perennial, tannin-containing forage legumes such as BFT into ruminant pastures and hay

enhances the sustainability and the potential profitability of livestock systems, while

improving ruminant performance, reducing GHG emissions, and enhancing ecosystems

services such as soil organic matter and C sequestration. The dissertation contains three

related studies examining (1) *in vivo* enteric CH_4 emissions by heifers grazing tannin-

containing legumes and non-tannin containing forages, including soil N and C

availability in agricultural plots; (2) an *in vitro* study of the rate and extent of fiber

digestion of a range of forage legumes, a grass and a forb, and (3) the construction of N

balance based on a controlled environment study of legumes with or without tannins

carrying out biological N_2 fixation, versus a non-fixing grass and forb. The N balance study summarizes N sources and sinks in simulated grazed pastures systems from establishment through eight harvest cycles, compressing approximately three years of forage production into a one-year intensive experiment.

Objective 1: Investigate effects on enteric methane emissions and soil quality in beef cattle pasture systems, comparing a perennial tannin-containing and a non-tannin legume with a grass (Chapter II).

Hypotheses 1: Grazing a CT-containing legume (BFT) or a non-tannin legume (CMV) will result in reduced enteric CH_4 emissions while enhancing N and C utilization due to greater legume forage digestibility relative to the grass. The CT in the BFT system may further reduce CH4 emissions, N mineralization and nitrification relative to CMV due to the presence of tannins, enhancing N and C retention in soils. Greater N and C stocks in CT-containing legume systems will reduce negative environmental impacts and increase soil health, potentially improving long-term farm profitability.

Objective 2: Determine *in vitro* fermentation kinetics and actual dry mater digestion of tannin-containing and non-tannin legumes, a grass and a forb and their isolated fiber to understand the influence of fiber and secondary compounds on hays fed to cattle (Chapter III).

Hypotheses 2: I*n vitro* digestibility of dry matter and organic matter, and fermentation kinetics parameters of whole forage as hay of six forage species and their isolated NDF fractions will be affected by the nutritive value and fiber digestibility of these ruminant feeds, and the presence of tannins in sainfoin (*Onobrychis viciifolia*), BFT and SB.

Objective 3: Compare N balances under controlled conditions over simulated long-term grazed pastures, including legumes with biological N_2-fixation as well as a non-fixing forb and grass species under a controlled environment, to evaluate the impact of N_2 fixation and plant secondary compounds such as tannins (CT and HT) on N cycling (Chapter IV).

Hypotheses 3: Nitrogen and C losses will be less under tannin- containing species due to a lessening of soil microbial processes (mineralization and nitrification). Thus, reducing NO_3^- leaching, and minimizing loss of N_2O while increasing C sequestration will result in greater environmental sustainability of legume-based systems through mitigation of agricultural N losses and GHG emissions.

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CHAPTER II

EFFECTS OF GRAZING PERENNIAL TANNIN-CONTAINING LEGUMES ON

ENTERIC METHANE EMISSIONS WHILE INCREASING N AND C

SEQUESTRATION IN BEEF CATTLE PRODUCTION SYSTEMS

ABSTRACT

The current study has demonstrated that grazing of the alternative forage legumes birdsfoot trefoil (*Lotus corniculatus* L.; BFT) and cicer milkvetch (*Astragalus cicer* L.; CMV), resulted in more sustainable beef production than grazing of meadow bromegrass (*Bromus biebersteinii Roem. & Schult*; MB). The legumes had greater feed quality and are able to fix their own nitrogen (N) from the atmosphere. Cicer milkvetch produced more dry matter and had greater forage quality than BFT and MB, likely resulting in greater nitrification rates and soil NO_3^- availability. Birdsfoot trefoil accumulates a limited concentration of condensed tannins, and tannins have been shown to increase soil N by slowing N mineralization and nitrification. Tannins bind with proteins in the rumen, and some tannins, like those in BFT, release protein at the low pH of the abomasum for gastric digestion. Tannins improve ruminant N retention, and commonly increase the ratio of fecal to urinary N. This can reduce N volatilization and the deposition of N in the soil in inorganic forms, while increasing soil organic N, increasing soil organic C. Greater fecal N reduces the probability of NO_3^- leaching or emission of nitrous oxide (N_2O), a potent greenhouse gas, from the pasture system. Greater organic C under grazing is likely explained by both manure and plant litter depositions. Indeed, in the

present study greater soil C sequestration was evident under MB and small burnet (*Sanguisorba minor* Scop.; SB), a tannin-containing forb. This effect was mainly detected in the uppermost (0-10 cm) soil layer where greater soil microbial activity responded to greater soil warmth and oxygenation, and greater turnover of fine roots and root C exudation. Methane (CH_4) is another potent GHG, and represents wasted food energy since reduced C is released to the atmosphere. Enteric CH_4 emissions were measured during grazing for individual animals for successive 24-h periods. Greater legume forage quality resulted in greater dry matter intake (DMI) per unit of respired enteric methane than for the grass. The rate of ruminal passage is greater for legumes because they have less fiber and therefore shorter retention in the rumen. No effect of BFT tannins on enteric CH_4 emissions could be distinguished. Reductions in enteric CH_4 emissions due to greater feed quality result in a reduced C footprint for beef production on legume pastures.

Keywords: legumes, tannins, nitrogen, carbon, methane, beef, sustainable.

1 INTRODUCTION

Ruminant production is the focus of public scrutiny because cattle and other ruminants are the major source of greenhouse gas (GHG) emissions from agricultural livestock production systems (Ripple et al., 2014). These GHG emission sources include methane (CH_4), nitrous oxide (N_2O), and carbon dioxide (CO_2). Methane is a GHG 28 times more potent than CO_2 (IPCC, 2013), primarily produced in the rumen through acetate metabolism, and exhaled through the mouth and nose as a normal byproduct of

digestion (Murray et al., 1976; Hook et al., 2010); and represents wasted food energy. The loss of enteric CH_4 to the atmosphere varies based on the ruminant species, geographical location, feed quality, feed intake, and the processing of the feed (Johnson and Johnson, 1995; Johnson and Ward, 1996). In western North American beef systems, 80% of GHG emissions were found to occur in the cow-calf phase. On a CO_2-equivalent basis, 63% of these GHG emissions were due to enteric CH_4, and 84% of enteric CH_4 emissions were due to mother cows (Beauchemin et al., 2010). Therefore, mitigation of GHG emissions from beef production during the cow-calf phase is crucial to reducing the GHG emissions of beef production systems.

Another GHG, nitrous oxide (N_2O), has a warming potential 265 times that of CO_2 (IPCC, 2013) and typically accounts for up to approximately 27% of total emissions from beef production (Beauchemin et al., 2010). Emissions of N_2O depend on abiotic factors such as soil temperature, aeration and soil water content. Urine and dung patches are significant sources of N_2O (Cai et al., 2017). The CH_4 emissions from manure and CO_2 emissions from other sources were irrelevant contributions to the GHG footprint of beef systems. Hence, decreasing enteric CH_4 and soil N_2O emissions from pasture systems while improving ruminant production is desirable both as a strategy to reduce global GHG emissions and as a means of improving feed conversion efficiency (Martin et al., 2010).

High quality forages with greater digestibility are expected to yield lower CH_4 emissions (Johnson et al., 2007) by reducing microbial fiber fermentation and increasing rate of passage from the rumen (Hook et al., 2010). Pasture legume species such as

birdsfoot trefoil (*Lotus corniculatus* L.; BFT) and cicer milkvetch (*Astragalus cicer* L.; CMV) have less fiber and greater protein and nonfibrous carbohydrate (NFC) concentration than forage grasses such as meadow bromegrass (*Bromus biebersteinii* Roem. & Schult.; MB), (MacAdam and Griggs, 2013; MacAdam and Villalba, 2015; Chail et al., 2016). Consequently, forage legumes are digested more rapidly, achieving greater nutrient intake and gains than forage grasses, with a concomitant reduction in land use. Even more, their biologically fixed N lessens economic and environmental impacts relative to grass-based beef finishing systems (Muir et al., 2014). While the perennial pasture legumes BFT and small burnet (*Sanguisorba minor* Scop.; SB) are non-bloating because of the tannins synthesized in their foliage, CMV, a non-tannin legume, is non-bloating because of its leaf morphology (Lees et al., 1982); these forages can all be grazed in pure stands.

Condensed tannins (CT) are plant secondary metabolites effective in reducing dietary energy loss (Cieslak et al., 2013) and CH_4 production (Woodward et al., 2001; Woodward et al., 2004) because they are toxic to protozoa (Jones et al., 1994; Min and Hart, 2003; Cieslak et al., 2012), rumen microbiota that generate hydrogen gas (Martin et al., 2010; Saminathan et al., 2016; Vasta et al., 2019). Tannins suppress the generation of acetate which contributes to methane synthesis in the rumen (Cieslak et al., 2013; Vasta et al., 2019). In addition to reducing CH_4 emissions, CT produced by BFT bind and precipitate excess plant proteins in the rumen, reducing their microbial digestion (Mc Sweeney et al., 2001), and allowing plant proteins to be released and digested in the abomasum, and component amino acids to be absorbed from the small intestine (Mueller-

Harvey, 2006; Waghorn, 2008; Aufrère et al., 2013). Many workers (Koenig et al., 2018; Lagrange and Villalba, 2019; Stewart et al., 2019) have reported an elevated ratio of fecal to urinary N in ruminant consuming CT-containing diets.

Depending on their physiological state, between 75 and 95% of the N consumed by ruminants becomes available for plant uptake via urine and feces (Haynes and Williams, 1993; Whitehead, 1995; Muir et al., 2014). Most of the N in urine from cattle is present as urea and other water-soluble organic molecules, which can rapidly be converted to plant-available forms of N (Whitehead, 1995; Menneer et al., 2003). By contrast, a greater proportion of the N in feces is bound in insoluble organic compounds that are converted to plant-available N via mineralization (Whitehead, 1995; Menneer et al., 2004). Partitioning of ruminant N waste toward feces has a favorable environmental impact since fecal N is retained as soil organic matter, increasing plant-available N stores, whereas urinary N is lost via volatilization as NH_3, and N_2O or may be leached into groundwater as NO_3^- with heavy precipitation or excessive irrigation (Waghorn, 2008; Woodward et al., 2009; Leip et al., 2015). The CT-protein complexes excreted in feces (Waghorn, 2008; Eckard et al., 2010) decelerate mineralization and nitrification rates, and inhibit soil microbes (Smolander et al., 2012; Clemensen et al., 2018), improving soil organic matter content and quality.

The aim of this study was to assess the beneficial effects of grazing CT-containing (BFT), and CT-free (CMV) legumes and grass on enteric CH_4 emissions and soil N- and C-stores. We hypothesized that these perennial legumes would reduce enteric

CH$_4$ emissions, and that BFT would impact N and C by reducing N mineralization and nitrification rates relative to both the non-tanniferous legume CMV and the grass MB.

2 MATERIALS AND METHODS

This field study was carried out at the USU Intermountain Irrigated Pasture Project in Lewiston, Utah, USA (latitude 41°56' N, longitude 111°52' W; 1374 m a.s.l.), according to procedures approved by the Utah State University Institutional Animal Care and Use Committee (application 2351).

2.1 Pasture design

Two soil series are present at the 6.6-ha study site: 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. Before planting, in August of 2012, soil samples were collected across the site and deficiencies of phosphorus and potassium were addressed. Three monoculture pasture treatments were replicated five times; each pasture replication was approximately 0.365 ha (64 x 57 m) (Figure 2-1). In the center of each 'Langille' BFT plot a strip 64 x 3 m of 'Delar' small burnet (*Sanguisorba minor* Scop.; SB) was established as a reference species for N$_2$ fixation. The other two pasture treatments were 'Monarch' CMV and 'Cache' MB. The four species (BFT, CMV, MB and SB) were broadcast seeded using a Brillion planter (Brillion Iron Works Inc., Brillion, WI), and planted at rates of 20, 34, 37 and 53 kg pure live seed ha^{-1}, respectively. Legumes were inoculated with the proper *Rhizobium* species

before planting to supply N, and grass pastures received 168 kg N ha^{-1} y^{-1} as 34-0-0

fertilizer in 2013, 2014, and 2015; in 2015, 56 kg/ha was applied in early June, mid-July

and early Sept. All pastures were sprinkler irrigated for 12 hours every 2 weeks during

the growing season at a rate of 3.8 mm/h to add a total of 46 mm of water per irrigation,

matching the available water-holding capacity of the soil.

2.2 Grazing trial

In 2015, 30 1-year-old heifers were sorted into three groups of 10 cattle each, with

similar total body weight (BW). Each group was randomly assigned to one of the three

treatments, BFT, CMV or MB. Heifers were dewormed and provided with ear tags to

reduce flies. Pairs of heifers from each group were randomly assigned to one of the five

replications of a given treatment, and that rotationally grazed the same 0.36-ha pasture

for the period from 6 July to 21 August 2015. Heifers were moved to an ungrazed

paddock within the same pasture every 3.5 days; fresh water and trace-mineralized salt

blocks (Morton iOFIXT T-M) were always available. The perimeter of each experimental

pasture was fenced using t-posts and electrified high-tensile wire, and the entire study

area was enclosed in a 5-wire high-tensile electrified fence. The heifers' initial body

weight averaged 443 ± 44 kg. The grazing period consisted of a 14-day adaptation period

prior to the first week of data collection. One heifer from each pair was assigned to

enteric CH_4 determinations and the other heifer was used for dry matter intake (DMI)

determinations.

2.3 Soil Sample Collection and Analysis

Soil samples were collected from 0-10 cm, 10-30 cm, and 30-60 cm depths on 24 June 2015, before grazing began, and on 1 September 2015, after grazing had ended. In the two upper soil layers (0-10 cm and 10-30 cm), two subsamples composited of six cores were taken across each pasture replicate. From the deeper layer (30-60 cm), two subsamples comprised of three composited cores were taken across each replicate pasture. Composited samples were mixed, transported to the laboratory in coolers, and immediately sieved to pass a 2 mm screen and extracted with 2M KCl (1:5 soil: solution w/v). Inorganic N pool size was calculated from NO_3^- and NH_4^+ in soil KCl extracts using a flow injection colorimetric method (Lachat N Autoanalyzer: QuickChem 8500). The soil moisture content of fresh subsamples weighing approximately 15-20 g was determined after oven drying at 105°C for 48 h. Samples were divided, with some stored at 4°C and the rest air-dried. Air-dried soil used for N and C analysis was finely ground and sieved to pass a 0.25 mm screen. Total soil N was determined by the dry combustion method using a Skalar Primacs[SN] Nitrogen Analyzer. Total soil C was determined by dry combustion and total inorganic C by acid dissolution using a Skalar Primacs[SLC] Carbon Analyzer; soil organic C was calculated as the difference.

2.4 Plant Sample Collection and Analysis

Available pasture biomass before and after grazing during the experimental period was determined non-destructively using a Farmworks (Feilding, NZ) rising plate meter (RPM) calibrated for each plant species. Thirty readings were taken before and after

grazing by walking in a "lazy W" pattern and averaged. Forage DM was assessed for the next paddock to be grazed and the most recently grazed paddock each week. A calibration curve was developed by cutting measured forage to ground level under the rising plate meter each week during the study period (MacAdam and Hunt, 2015). Calibration samples were collected from a range of heights, and a linear regression of herbage dry mass on RPM readings was used to determine pasture production and forage disappearance.

Samples for forage nutritive value composition were collected by clipping grazable forage (including weeds) to grazing height at random locations across the next paddock to be grazed. Pure samples of each treatment species were also collected in the same manner from the next paddock to be grazed in each pasture each week. Plant samples were frozen under dry ice in the field and stored at -20°C until freeze-dried. Samples were ground to pass a 1-mm screen using a Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA) and stored in sealed plastic bags until chemical analysis. Forage nutritive value samples were analyzed by near infrared spectroscopy (NIRS), and pure plant species samples were analyzed for condensed tannins using the butanol-HCl acetone method of Grabber et al. (2013).

Pure herbage samples of all forage species were collected on 17 June 2015 and 10 August 2015, and analyzed by mass spectrometry to determine stable N isotope (^{15}N) composition. Variations in the natural abundance (NA) of ^{15}N were used to estimate the fractional contribution of N_2-fixation to N concentration of the legumes BFT and CMV. In this study, SB was a non-N_2 fixing reference forb with rooting depth and seasonal

growth characteristics similar to BFT which was used to quantify plant-available soil N.

It was assumed that no differential fractionation of N isotopes occurred during uptake of

soil N by SB and BFT. The analysis of N and $^{15}N/^{14}N$ ratios in June and August reflected

N_2 fixed in the whole plant (Heichel et al., 1981). To quantify the discrimination between

^{14}N and ^{15}N that occurs during N_2 fixation (Shearer and Kohl, 1986) in BFT and CMV,

inoculated BFT and CMV were established in 2-gal. pots of washed sand watered daily

with a N-free nutrient solution (Bergersen and Turner, 1983) to ensure plants were

completely dependent on N_2 fixation and account for ^{15}N discrimination in the N_2-fixing

plant (Evans, 2001).

Thus, $\delta^{15}N$ = $\dfrac{R\ sample - R\ standard}{R\ standard} * 1000$ $\qquad$ (Shearer and Kohl, 1986)

where:

R = $^{15}N / (^{14}N + ^{15}N)$

R standard = R air = 0.3663 atoms % ^{15}N

$\delta^{15}N$ = Parts per thousand deviation from the $^{15}N/^{14}N$ ratio of atmospheric N_2

Proportion of N fixed (P_{fix}) = 100 $(x - y) / (x-c)$ (Amarger et al., 1979; Kohl et al., 1980)

where:

P_{fix} = the proportion of BFT and CMV nitrogen derived from N_2 fixation,

x = the mean $\delta^{15}N$ of the total N of the non-N_2 fixing reference plant (SB) where N

requirements were obtained from the pool of soil mineral N,

y = the mean $\delta^{15}N$ of the shoot N of BFT and CMV samples,

c = represents a measure of the isotopic fractionation which occurs during N_2 fixation and is derived from the $\delta^{15}N$ of the total N of BFT and CMV plants obtained by fixation. This value was -4.32‰ for BFT and -1.34‰ for CMV.

The N_2 fixed by a single legume species was the the product of P_{fix} and total plant N (kg ha^{-1}).

Extreme care was taken to avoid cross-contamination among samples while weighing samples for ^{15}N enrichment determinations.

2.5 Dry Matter Intake Determinations

Dry matter intake (DMI) of grazing animals was determined using two different approaches: 1. By pasture DM disappearance calculated as the difference between weekly RPM measurements of pre- and post-grazing dry matter combined with the total grazing area allotted to each pair of cattle each week, and weekly interpolation of pre- and post study period body weight, and 2. Prediction of voluntary feed intake based on NIRS data for CP, TDN and ADF for grass, and data for NDF and NDFD for legumes, using the equations of Moore and Undersander (2002). Conversion of DMI data from a percent BW basis (Moore and Undersander, 2002) to a kg DM d^{-1} basis used the same weekly interpolation of pre- and post-season body weight used for pasture DM disappearance.

2.6 Ruminant Methane Emissions

2.6.1 Enteric Methane Collection

Enteric methane of heifers was determined using the SF_6 trace gas technique (Johnson et al., 1994; Johnson et al., 2007). Before grazing began, fifteen heifers selected for CH_4 sampling were trained during a 30 day period to wear halters and PVC canisters. Canisters were fitted under the chin of the heifers and attached to halters at both ends.

During the training period, a brass permeation tube with a known release rate of sulfur hexafluoride (SF_6) was placed in the reticulorumen of each selected heifer using a bolus gun. The SF_6 served as an internal standard for respiration volume. The SF_6 release rate of each permeation tube was assessed gravimetrically during six weeks of incubation at 39°C, and the mean permeation tube SF_6 release rate for this study was 0.79 ± 0.03 mg day^{-1}.

Enteric CH_4 was collected for four successive days on two replications of the three pasture treatments each week for five weeks. From weeks 1 to 5, reps 1 and 2, 3 and 4, 5 and 1, 3 and 4 and 2 and 5, respectively, were evaluated. During each sampling day, each heifer in these reps was fitted with a halter and evacuated canister. Canisters were 10 cm in diameter and 28 cm long schedule 40 polyvinyl chloride (PVC) canisters with PVC slip caps attached to both ends with primer and solvent cement; canister volume was approximately 2.5 liters. Canisters were fitted with Swagelok ball valves and quick connect fittings. Canisters were evacuated to a tension of 0.250 psi or less using a diaphragm vacuum pump (Vacuubrand Model MZ2NT, Wertheim, Germany) and an in-line digital pressure meter (Druck, Model DPI 705). Halters were fitted with a 50-cm

length of 125 µm ID x 1/16" OD U160 capillary tubing (IDEX, Oak Harbor, WA, USA)

that connected a filtered inlet above the mouth and nose to a quick connect fitting near

the chin. To begin collection, evacuated canisters were connected to the capillary tubing

on the halters, their ball valves opened, the time noted, and after approximately 24 h,

their ball valves were closed, the time noted, and canisters were disconnected from the

collection system and returned to the lab. After 24 hours, acceptable final tensions in

canisters were 0.25 to 0.67 atm. Tensions above or below that range indicated a leak or

blockage, respectively (Johnson et al., 2007).

Before field collection began, canisters fitted with capillary tubing systems were

placed in pastures to determine if there was significant background SF_6. During each day

of each collection period, control canisters were placed in ungrazed sections of each

treatment pasture. The inlet was positioned on top of a fence post at 1.5 m height, and

used to correct values obtained from cattle for ambient CH_4 (Williams et al., 2011).

2.6.2 Methane Analysis

The tension remaining in sample canisters at the end of the 24-h collection period

was recorded, and canisters were pressurized to 1.1 atm with high-purity N_2 gas and the

exact dilution pressure was recorded. Samples were extracted by connecting a male quick

release valve fitted with a septum to the female quick release connection on the canister.

The canister's ball valve was opened, and a 20-gauge needle attached to a 20 mL syringe

was filled with a 15- to 18-mL gas sample. The gas aliquot in the syringe was transferred

to pre-labelled 5 mL evacuated glass vials (Model 838 W, Labco Limited, Lampeter, UK)

fitted with a septum. Gas samples were analyzed for CH_4 and SF_6 concentrations at the Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, Alberta, Canada.

This methane method uses SF_6 to correct CH_4 for respiration volume because both gases are exhaled from the rumen at once and mixed with ambient air at the same dilution rates. Therefore, the CH_4 emission rate was calculated as the product of the known release rate of SF_6 and the ratio of CH_4 and SF_6 amounts found in the sample as follows. Enteric CH_4 emission was expressed as grams of CH_4 per head per day and per kg of DMI based on disappearance of forage from pastures.

CH_4 emission rate (g d^{-1}) = SF_6 release rate (g d^{-1}) x [g CH_4/g SF_6]

(Johnson et al., 2007)

2.7 Statistical analysis

Soil total N, organic N, total C, inorganic C, organic C, extractable NH_4^+ and extractable NO_3^- were analyzed with a repeated measures design with species (treatment), week and depth as fixed factors. Random effects were replication, species*block and week*species*block.

Pasture available biomass (pre-grazing, post-grazing and their difference), nutritional composition (DM, CP, aNDF, ADF, ADL, NFC, Fat, TDN, DDM, NDFD and ash), forage N concentration (pre-grazing, post-grazing and their difference), forage ^{15}N from samples collected before grazing began and after it ended, CT in forages, fecal

output, DMI, CH_4 emissions per animal per day and on the basis of DMI were analyzed using a repeated measures design with species (treatment) and week as fixed factors and replication and species by replication as random effects.

The covariance structure that yielded the lowest Bayesian information criterion was used for repeated measures. Analyses were conducted using SAS PROC GLIMMIX (SAT/STAT 15.1, SAS Institute, Cary, NC). Least squares means (LSMeans) were compared pairwise using the Tukey-Kramer test adjusted for multiplicity when the overall test for treatment effect was significant ($P \leq 0.05$). Assumptions of homoscedasticity of variance and normality were tested using studentized residuals. Variables like soil extractable NH_4^+, soil extractable NO_3^- and shoot CT concentrations were transformed to their natural logarithm in order to meet these assumptions. Reported LSmeans and standard errors estimated by the model were back-transformed from the log scale. Back-transformation of standard error was done by the delta method.

3 RESULTS

3.1 Climate data

Temperature, precipitation and evapotranspiration demand data for Lewiston, UT in 2015 (Figure 2-2) were provided by the Utah State University Climate Center Climate Database Server, which reports daily evapotranspiration estimated by the ASCE standardized Penman-Monteith method (ASCE-EWRI, 2005).

3.2 Soil nitrogen and carbon

For a given date and soil depth, soil organic N generally did not differ among

forage species (Table 2-1) but decreased with increasing soil depth for a given species

and date ($P<0.0001$). Soil organic N was greater before than after grazing ($P=0.0006$)

although no significant differences were observed before and after grazing for the 10-30

and the 30-60 cm soil layers. Soil organic N in the upper soil layer (0-10 cm), however,

was greater before than after grazing, causing a significant depth by date interaction

($P=0.0013$).

Species, depth and date had significant effects on extractable NH_4^+ over the

growing season (Table 2-1). Ammonia was greatest for SB soils, least for BFT, and

intermediate for MB and CMV soils ($P<0.10$). Ammonia was greater at 0-10 cm than at

10-30 cm or 30-60 cm ($P<0.0001$). In the middle soil layer (10-30 cm), NH_4^+ was greater

for CMV than BFT, and intermediate for MB and SB ($P=0.0016$), creating a species by

depth interaction. Soil NH_4^+ was always greater after than before grazing ($P=0.0036$).

Soil NO_3^- was significantly ($P<0.01$) affected by species, depth and date (Table 2-

1). There was more NO_3^- in BFT, CMV and MB than in SB soils regardless of depth and

date. Soil NO_3^- decreased from the shallowest (0-10 cm) to the middle oil layer (10-30

cm) and did not differ for 0-10 and 30-60 cm depths. Nitrate was also greater after than

before grazing. Before grazing, soil NO_3^- at 10-30 cm was greater for CMV than SB, and

MB and BFT were intermediate. Following grazing, NO_3^- was less for SB than for BFT,

CMV and MB at all depths.

Total soil C did not differ among species or between dates (Table 2-2) but was strongly affected by depth ($P<0.0001$), with greater values detected in the upper and lower soil layers (0-10 and 30-60 cm, respectively) than in the middle layer (10-30 cm). In pre-grazing soils, MB had greater total soil C at 10-30 cm than BFT and CMV soils but did not differ from SB, causing a significant species by depth interaction ($P=0.0085$). For the 10-30 cm soil depth, total soil C was increased by grazing ($P=0.0217$), resulting in a depth by date interaction.

Inorganic soil C was similar among species within dates but differed by depth ($P<0.0001$; Table 2-2), with the deepest soil layer (30-60 cm) reaching the greatest values, followed by the middle layer, and least values occurred in the shallowest soil layer (0-10 cm). A forage by depth interaction occurred due to differences in inorganic soil C in the deepest layer (30-60 cm), where MB and CMV were greater than BFT, while SB was intermediate.

Soil organic C was also similar among species and within dates but decreased with increasing soil depth ($P<0.0001$; Table 2-2). In pre-grazing soils, MB had greater organic soil C than BFT and CMV soils at 10-30 cm and SB was intermediate. A depth by date interaction was observed, mainly driven by greater soil organic C in the 10-30 cm soil layer before than after grazing.

3.3 Forage availability

Both pre- and post-grazing pasture DM was greater for the two legumes than for the grass (Table 2-3). Pre-grazing DM was greater for CMV than BFT, and BFT was

greater than MB ($P<0.0001$). However, there was a significant week to week variation among species for pre-grazing DM. Forage disappearance was greater for CMV than for BFT and MB. Pasture DM utilization was 43, 51, and 54% for BFT, CMV and MB, respectively.

3.4 Diet composition

The greatest values for aNDF ($P<0.0001$), ADF ($P<0.0001$), fat ($P<0.0001$) and ash ($P<0.0001$) were found in MB (Table 2-4). NDF and ADF were greater for BFT than for CMV. Cicer milkvetch had the greatest CP ($P<0.0001$), NFC ($P<0.0001$), TDN ($P<0.0001$) and DDM ($P<0.0001$). These characteristics were all greater for BFT than for MB. Birdsfoot trefoil had the greatest ADL concentration, and CMV ADL was greater than MB ADL ($P<0.0001$). The NDF digestibility was less for BFT than for CMV and MB, which did not differ ($P<0.0001$).

The tannin concentration was measured in shoots of all forages, including CMV and MB (Figure 2-3) which are not reported to contain condensed tannins. Mean tannin concentrations in BFT differed among weeks ($P<0.0001$), with greatest values in the third week of sampling and least values in week 5.

3.5 N$_2$ fixation in legumes pastures

Smaller values for $\delta^{15}N$ indicate N$_2$ fixation, and in June 2015 legumes had less ($P<0.0001$) $\delta^{15}N$ than SB or the grass, MB (Table 2-5) confirming N$_2$ fixation before grazing began. Values for $\delta^{15}N$ were less for all species in September 2015, after grazing had ended. Birdsfoot trefoil had lower values than MB, while both CMV and SB were

intermediate. Legume $\delta^{15}N$ declined during grazing. Cattle did not move between pastures treatments, so N in dung and urine was from the plant species planted in each pasture. The proportion of N derived from N_2 fixation (P_{fix}) in BFT and CMV did not vary by species or season, with both species averaging about 50% fixed N_2 before and after grazing. Both legumes species declined to about half the amount of N fixed after grazing compared with pre-grazing pastures.

3.6 Total N, ^{15}N and tannin concentrations in feces

Data for N concentration, $\delta^{15}N$ and CT concentrations in feces are reported in Table 2-6. Cicer milkvetch and MB were not expected to contain tannins, and very low values were likely due to plant phenolics other than tannins.

3.7 Dry matter intake

Dry matter intake calculated (1) from NIRS parameters (g intake kg BW^{-1}) and the BW in kg of cattle on each pasture treatment, and (2) from forage disappearance (Table 2-3) calculated from the difference between pre- and post-grazing pasture DM, the area grazed and the time spent grazing, resulted in similar values for DMI (Figure 2-4) and demonstrated that DMI differed in the order CMV > BFT = MB (P=0.002).

3.8 Enteric methane emissions

Averaged across weeks, the daily gross CH_4 emission (g d^{-1}) differed among the different diets in 2015 (Table 2-7). Cattle grazing BFT and CMV emitted less enteric

methane than cattle grazing MB pastures. When enteric methane emissions were expressed per unit of DM intake, the results were the same (Table 2-7).

4 DISCUSSION

4.1 Nitrogen and C stores

Grazing animals play a key role in the livestock systems through the circulation of nutrients such as N and C within the environment. Indeed, roughly 80% of ingested nutrients are returned to the soil via manure and remain in the ecosystem (Temperton et al., 2007; Muir et al., 2014). In the present study, feces from both legume treatments contained greater N concentrations than MB feces, thus resulting in potentially greater manure N_2O emissions similar to observersations from confinement dairy systems (Little et al., 2017). However, fecal N distributed directly onto the soil surface in rotationally stocked pastures quickly becomes incorporated and is converted to NH_4^+ at a relatively slow rate, retained in the soil, and contributes to accumulation of SOM (de Klein and Eckard, 2008). The tannins present in feces from cows consuming legumes such as BFT can also decrease the rate of N mineralization (Eckard et al., 2010; Stewart et al., 2019), further reducing the potential for N_2O emissions in tannin-rich dung patches.

Apart from the influence on soil nutrient availability and soil physical properties, manure deposition can also affect biological activities and plant growth (Haynes and Williams, 1993; Cai and Akiyama, 2016), and indirectly increase sequestration of atmospheric C from greater litter input (Paustian et al., 1997a; Paustian et al., 1997b;

Conant et al., 2001). In this study, the upper 30 cm of the soil had greater organic C following grazing, likely explained by both manure and plant litter depositions.

Nitrogen is an essential nutrient for plant growth and development, and plant growth is often limited by the availability of N in the ecosystem. The transformations of organic N to NH_4^+ and NO_3^- are central processes in the internal soil N cycle mediated by soil microbes. Ammonium is produced through mineralization of soil organic N, and can be rapidly converted to NO_3^- by nitrification (aerobic processes), increasing the likelihood of N loss from agricultural ecosystems through NO_3^- leaching (Schlesinger, 2009) or N_2O atmospheric emissions (Hofstra and Bouwman, 2005; Robertson and Vitousek, 2009; Hu et al., 2015). In the present study, N was supplied as NH_4^+ through mineralization of soil organic N, biological N_2 fixation by legumes systems and fertilization of MB pastures with ammonium nitrate. The available NH_4^+ by fertilization was probably consumed in less than a month, and then soil NH_4^+ was supplied by mineralization of soil organic N in the grass systems (Habteselassie et al., 2006; Habteselassie et al., 2013). Nitrogen from pastures was also recycled as urine and feces.

Both the CP concentration and the DM production of CMV was greater than these variables for BFT, which were greater than for MB, so it could be expected that greater crude protein in the diet would add more N to the CMV system. Previous researchers have reported that dietary N concentration affects the N concentration of urine rather than feces. Urinary N excretion can vary from about 45 to 80% of total excreted N (Ledgard and Steele, 1992; Whitehead, 1995). Urine volume and N concentration were not measured in the present study, but in a study of cattle fed hay of these species, urine

accounted for 49, 60, 48 and 15% of N excretion from cattle fed BFT, CMV, MB, and SB, respectively (Stewart et al., 2019). These proportions are reflected in data for soil NO_3^- accumulation at the end of the grazing study, with greater values for MB and the legumes, and lesser values for SB.

The balance of N excretion was in feces, at 51, 40, 52 and 84% for BFT, CMV, MB, and SB, respectively (Stewart et al., 2019) but there is no comparable elevated value of NH_4^+ for SB. Reduced partitioning of ingested N to urine and more to fecal excretion is likely due to the hydrolyzable tannins present in SB (Stewart et al., 2019). Tannins are known to improve ruminal protein use and shift the partitioning of N from urine to feces (Barry et al., 2001; Misselbrook et al., 2005) which ultimately slows the release, leaching and emission potential of N in pastures (Koenig et al., 2018; Lagrange and Villalba, 2019) resulting in greater agricultural sustainability (Ramirez-Restrepo and Barry, 2005). The CT concentration of BFT ranged from 1-2%, low enough that it did not suppress the partitioning of N to urine in this study, while on a diet of SB alone, dietary protein is excessively restricted by HT (Smolander et al., 2012). In both BFT and CMV, the pasture concentrations of NFC provide an additional nutritional benefit to ruminants, reducing GHG emissions and urinary N excretion from ruminants (Villalba et al., 2019) while enhancing ruminant production relative to perennial forage grasses such as MB.

There was some concern that, under grazing conditions, soil N would accumulate to greater levels for legumes than for a fertilized grass (Conant and Paustian, 2002), since legumes have high crude protein concentrations and have access to a dedicated source of N (Jarecki and Lal, 2003). However, there were no differences in soil N between the

grass and the legumes at the end of the season. Before grazing, soil N was greater in the shallowest soil layer; and decreased deeper in the soil profile, likely because of root activities, while at the end of the season, inorganic N was well-distributed through the 60-cm-deep soil profile. As forages are grazed, roots are pruned because less photosynthate is available for their maintenance, and root turnover can contribute to soil organic N through mineralization; the increase seen in NH_4^+ in deeper soil layers at the end of the grazing season may be due to this grazing study period root turnover. The added organic matter contributes carbohydrates and nutrients that serve as an energy source for soil microbes (Ta et al., 1990), thus increasing microbial biomass and activity (Bardgett et al., 1998).

The leaf litter of legumes that have high digestibility when consumed by ruminants are also readily decomposed by soil microorganisms due to a low C:N ratio, resulting in greater organic N mineralization rates. The reduced concentration of NH_4^+ in BFT at the 10-30 cm soil depth before grazing began could be due to the presence of tannins which inhibited soil N mineralization rates (Schimel et al., 1998; Bradley et al., 2000; Kraus et al., 2004). Management of BFT for spring regrowth requires stands to rest and regrow during fall, so there is significant litter accumulation that would have become incorporated into the soil over the winter. Ouyang (2016) concluded that the application of organic N through plant residues and feces increases the diversity of the microbial community and N mineralization. Previous workers have documented the impact of BFT CT on slowing N mineralization rates and minimizing N loss (Misselbrook et al., 2005; Powell et al., 2009). The relatively low levels of soil NH_4^+ suggest plant growth is able to

consume mineralized NH_4^+ more readily than NO_3^-, or alternatively that nitrification is moving most mineralized N into the NO_3^- pool.

Cattle were not put on pastures until July 6, and there was considerable accumulated pasture DM by that date. Soil NO_3^- pools were greater for CMV than SB, but not statistically different from BFT and MB both before and after grazing. Scherer-Lorenzen et al. (2003) and Palmborg et al. (2005) found that soil NO_3^- concentrations were positively correlated with biomass under pure legume communities and negatively correlated under non-legume communities. Cicer milkvetch produced more pastures DM than BFT and MB, suggesting that the elevated NO_3^- resulted from elevated nitrification rates under CMV (Cadisch et al., 1994). Nitrate results from the activity of microbial nitrifiers, and their activity may be suppressed by the presence of tannins in plant residues and manure (Baldwin et al., 1983; Adamczyk et al., 2013; Winder et al., 2013). The apparent intermediate nitrification under BFT and MB may be due to the CT present in BFT litter (Clemensen, 2018) and greater cell wall contents in MB that immobilized N (Hooper and Vitousek, 1998). Overall, tannins help conserve soil N in pastures systems, reducing NO_3^- leaching and N_2O emissions from the whole system, reducing GHG emissions and the C footprint. Elevated NO_3^- concentrations at the greatest depth at the end of the grazing season suggests significant NO_3^- movement in these pasture soils. Although our irrigation management was devised to minimize excess water application, some downward movement of excess NO_3^- would occur as the soil profile was filled with water.

The crude protein concentration of the forages in this study ranges between 19% for the grass and 22-26% for the legumes. The DM production of these forages was in the same order: CMV > BFT > MB. The legumes relied on N_2 fixation while the grass was fertilized with ammonium nitrate fertilizer. In general, available soil N (NH_4^+ and NO_3^-), the fraction plants can take up and assimilate, is associated with greater DM availabilities in agricultural systems during the growing season (Whitehead, 1995). The present study can provide insight into N uptake or movement in the soil in the period between growing seasons. If the post-grazing soil inorganic N status indicates available N going into the winter, and the difference between these values and pre-grazing soil N status the next spring is a measure of net soil N, it appears that MB and SB consumed (or lost) NO_3^- from the 10-30 and 30-60 soil layers, as did BFT, but the legumes also contributed new NO_3^- in the upper (BFT) or 10-30 cm soil layer (CMV). It appears that much of the NH_4^+ from mineralization of sloughed roots and incorporated ruminant waste that was present after grazing throughout soil profiles in September was consumed by early July of the following year. However, the differential in DM production of these three forages does not appear to be a function of available soil N.

Biological N_2-fixation by legumes could replace industrial N fertilizers used to fertilize grass pastures by planting mixtures of grasses and legumes (Schlesinger, 2009), thereby reducing the consumption of fossil fuels involved in plant production and N losses to the environment (Muir et al., 2014). Efficient legume-rhizobia symbioses can provide sufficient N to partly or entirely replace the need for N fertilization. Furthermore, there is substantial evidence that fixed N is transferred from legumes to neighbouring

species (Mulder et al., 2002; Spehn et al., 2002) as was seen in this study as an intermediate level of $\delta^{15}N$ of SB, which was included in BFT treatments as an indicator of N transfer from legumes to non-legumes. Yield was not measured for SB in this study, but soil under this part of the pasture was more depleted of mineralized N (NH_4^+) than that of the other forages. Since the only source of new N in the SB strip in the center of BFT pastures was urine and feces from grazed BFT herbage, it is reasonable to surmise that BFT N fixation supported SB growth. In the present study, P_{fix} data indicated that BFT and CMV derived at least half of their N from N_2 fixation. The N-fixing bacteria present in the root nodules of legumes appear to be capable of providing all the N needed for pasture plant growth, including that of companion species, but nodule N_2-fixation can decreases if soil N becomes available from excretion, minimizing the creation of excess N in pasture systems (Streeter and Wong, 1988; Menneer et al., 2003), and thus creating an environmentally and economically sustainable agricultural production systems.

Soil organic matter (OM) constitutes the dominant C stock in terrestrial ecosystems (Conant, 2010). Under proper grazing management, perennial forages are known to recycle about 90% of their C back to the soil (Paustian et al., 1997b; Guo and Gifford, 2002; Conant et al., 2003), thereby removing C as CO_2 from the atmosphere through photosynthesis and enhancing soil quality. Indeed, perennial forages contribute to soil C sequestration through large root systems that expand with shoot growth but are pruned by the plant after harvest or during dormancy (Bolinder et al., 2007). The amount of C stored in soil is the difference between plant litter (Paustian et al., 1997b), sloughed root organic matter, and decomposition by soil microbes which ultimately release the C

back into air as CO_2 (Guyader et al., 2016). Thus, soil C storage not only varies with grazing management (Conant et al., 2001) but also among forage species (Guo and Gifford, 2002). Fundamentally, the production of perennial legumes is constrained by the availability of water in the system, and by the genetic potential for DM production of the plant material. In the present irrigated forage production study, the tap-rooted legume BFT and the rhizomatous CMV had less soil organic C (SOC) at 10-30 cm before grazing began than MB, a fibrous-rooted grass. Although root biomass was not measured in the present study, it is likely that greater root biomass and total root C accumulated in the soil of MB, as was seen in our column study, resulted in enhanced soil C sequestration from C exudation by the roots and greater turnover of fine roots (Smith and Paul, 1990; Guo and Gifford, 2002; Shahzad et al., 2015). Indeed, Clemensen (2018) reported greater C storage and N immobilization with the incorporation of fibrous root systems such as grasses. The enhancement of SOC in the uppermost soil layer was not altered by grazing and was likely due to greater soil microbial activity in this warmer and more oxygen-rich layer, as well as to greater nutrient concentrations from the accumulation of forage residues (Zhou et al., 2007), proliferation and turnover of fine roots (Conant et al., 2003) and waste deposition.

4.2 Enteric Methane Emissions

Reduced enteric CH_4 emissions have been associated with reduced environmental pollution and enhanced animal nutrition because CH_4 represents an energy loss of 2 to 12% of the gross energy consumed in the diet (Johnson et al., 1993). In the present study, enteric CH_4 (g d^{-1}) emissions were less for cattle grazing BFT and CMV than for cattle

grazing MB (MacAdam et al., 2016). Similar results were found in a 2014 study of pregnant beef cows carried out by Pitcher (2015).

It is known that animals consuming forages low in fiber and high in non-fibrous carbohydrates such as CMV and BFT produce lower CH_4 emissions than animals consuming grass or more fibrous feeds (Dewhurst, 2013). These responses are related to DMI (Pinares-Patiño et al., 2009; Pacheco et al., 2014; Lima et al., 2016) which is negatively correlated with forage fiber concentration (Van Soest, 1994; Griggs et al., 2010). Thus, DMI is greater for ruminants fed legumes than grasses (Van Soest, 1965) since legumes that have more crude protein and less fiber than grasses can be digested more readily (Smith et al., 1972; Wen et al., 2002).

In the present study, CMV had greater forage quality than BFT, and BFT had greater forage quality than MB, with forage quality considered to be greater crude protein, reduced fiber, reduced lignin and greater non-fibrous carbohydrates concentrations (Minson, 1985; Griggs et al., 2010). Forage quality is also better for high-protein forages that include moderate concentrations of CT (Ramírez-Restrepo and Barry, 2005; MacAdam and Villalba, 2015), more DDM, greater fiber digestibility and greater DMI. Reduced fiber digestibility is associated with greater ADL, as for BFT relative to CMV in this study. In this field study, the CT concentration of BFT averaged 15.3 g kg^{-1} DM, with CT concentration peaking July 20 and decreasing later in the season (Wang et al., 2015). These values are consistent with the CT concentrations of BFT reported by Grabber et al. (2015) of 1.4 to 3.2% for a range of North American and Mediterranean cultivars. The CT of BFT in the present study was below the threshold concentration of

CT (50 g kg^{-1}) cited by previous studies (Barry and McNabb, 1999; Waghorn, 2008; Wang et al., 2015) as potentially limiting of feed intake and decreasing DDM in ruminants.

Dry matter intake is one of the most important factors impacting CH_4 emissions by ruminants (Jiao et al., 2014). Cattle grazing CMV had 37% greater DMI than cattle grazing BFT, and 62% greater DMI than cows grazing MB. The reduced intake of grass systems is because a greater NDF concentration slows rumen digestion and therefore the emptying of the rumen, which is required before more forage can be grazed. While MB has greater NDF digestibility than other grasses (MacAdam and Griggs, 2006), and is therefore likely to have minimal enteric CH_4 emissions among the adapted cool-season grasses, diets with more grass are expected to have greater enteric CH_4 emissions than diets with more legumes. Legume diets, in contrast, have greater nutritional quality, less fiber, and therefore reduced retention digesta times in the rumen, so less CH_4 is generated by the fermentation of fiber (Moss et al., 2000; Guyader et al., 2016). The fiber that legumes do contain has more-concentrated lignin, and fiber bundles are smaller and shorter, which means that they are more likely to leave the rumen undigested and pass through the gastrointestinal track and into waste intact (Van Soest, 1994). This lignified fiber contributes to fecal waste and to the C that is retained by the soil until it is mineralized by soil microbes. The tannins in BFT are miniminal but those that pass intact from ruminant digestive systems will be found in feces and will slow the mineralization of SOM. There is no evidence in this study that less NH_4^+ was generated by the end of the season in BFT and CMV systems relative to the MB system, but this is likely to be

because production of shoots was greater, production of roots likely less, but

mineralization of legume fiber was reduced compared the mineralization of grass fiber.

The enhanced efficiency of legume digestion by ruminants, along with the

enhanced concentration of non-fibrous carbohydrates in legumes, may favor propionate

over acetate short-chain (volatile) fatty acid production in the rumen, which is considered

a competitive pathway for hydrogen use (Moss et al., 2000; Hassanat et al., 2013; Vasta

et al., 2019); and also reduces the amounts of C available for the production of CH_4

(Daniels et al., 1984; Whitelaw et al., 1984), contributing to both reduced gross CH_4

production and net CH_4 yield (Hart et al., 2009; Guevara-Ballesteros, 2019). In contrast,

fermentation of a more fibrous diet, such as that of MB, results in more acetic acid and

increased CH_4 production in the rumen as was seen in our study, since acetate is the main

component in CH_4 production (Johnson and Johnson, 1995; Ominski and Wittenberg,

2004). More CH_4 production during rumen digestion directly increases the concentration

of CH_4 exhaled through the animal's mouth and nose, representing a greater loss of gross

energy (Mountfort et al., 1982; Hook et al., 2010).

The effect of BFT CT on enteric CH_4 emissions could not be distinguished in this

study from the overall effect of forage quality (Guglielmelli et al., 2011; Rufino-Moya et

al., 2019), in fact the daily enteric emissions per head of cattle grazing BFT and CMV did

not differ. On the basis of DMI, the CT in BFT may have offset the greater forage quality

of CMV through a direct reduction in CH_4 generation in the rumen (Schofield et al.,

2001). Indeed, it has been demonstrated *in vitro* that the type (procyanidin-rich) and

concentrations of CT accumulated in BFT are important factors affecting enteric CH_4

production (Mangan, 1988; Szumacher-Strabel et al., 2011; Hatew et al., 2016). In studies carried out in New Zealand, BFT tannins reduced enteric CH_4 emissions (Woodward et al., 2004) while studies carried out in the Mountain West, where tannin accumulation is relative reduced, BFT did not reduce CH_4 emissions (Guevara-Ballesteros, 2019; Stewart et al., 2019). The methodology required for daily collection of enteric CH_4 collection in pastures is challenging for the cattle as well as for researchers. However, results collected in 2015 from these yearling beef cattle were consistent with results for heavily pregnant beef cattle collected from the same pastures in 2014 (Pitcher, 2015).

Finally, enteric CH_4 production observed in this study was considerably less than the values reported by Chung et al. (2013) using fresh alfalfa and sainfoin (25.7 vs 26.1 g kg^{-1} DMI, respectively) or the 19.9 g kg^{-1} DMI reported by Woodward et al. (2004) for BFT. The two methodologies used to estimate forage DMI were independent, with one based on physical measurement of change in the forage DM of pre- and post-grazed pastures, and the other based on NIRS analysis of forage nutritive quality characteristics of the three forages, so we are confident in our DMI data. Field measurement of enteric methane emissions is notoriously variable, especially because cattle are wearing canisters that impinge on grazing, and the cattle must be handled every day. We sought to reduce the stress on our enteric test animals by only collecting enteric CH_4 from them for one week out of every three, and by only using these animals for enteric CH_4 collection, while using the BW data of the other animal on the same pasture to generate DMI data.

5 CONCLUSIONS

In conclusion, the use of perennial legume pastures such as BFT and CMV which fix their own N from the atmosphere, and are more digestible due to greater crude protein and non-fibrous carbohydrates and lower fiber concentrations, result in greater intake than grass pastures, and can lead to reduced environmental impacts and improved ecosystems services through more efficient N use and reduced enteric CH_4 emissions. Tannin-containing forages like BFT can contribute to reduced urinary N and improved rumen synchrony of carbohydrate and protein in the rumen as well as greater synchrony of N mineralization with plant N uptake in the soil, and thereby reduce ecosystem nutrient losses. This contributes to greater soil C sequestration and reduced GHG emissions from ruminant production systems as well as enhanced soil quality.

Grasses such as MB with elevated fiber digestibility relative to other cool-season grasses, and forbs such as SB with elevated hydrolysable tannins, have substantial potential for sequestration of atmospheric C as evidenced by equal soil organic C accumulation over the 2015 grazing season. These species in mixtures are likely to have positive associative effects beyond their individual attributes. In monocultures or mixtures, they are likely to enhance the resilience of ruminant production systems in the face of climate variability, long-term adaptation to changing climates, and thereby lead to increased production, biodiversity, and greater economic returns. Though soil C sequestration occurs slowly, over many seasons, western pasture systems employing productive, nutritious forages under irrigation on alkaline soils may show measurable results within years rather than decades.

Finally, reductions in enteric CH_4 emissions due to the incorporation of greater feed quality such as legume pastures can clearly have a significant impact on total C footprint of beef production, although greater CH_4 emissions in MB systems can be offset to some extent by enhancing soil C reserves. Therefore, perennial forages systems must be analyzed as holistic systems that take into account the trade-offs between feed resources used, animal performance, enteric CH_4 production, and soil C reserves.

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TABLE 2-1 Soil nitrogen (N) concentrations (g N kg^{-1} soil) and inorganic N (KCl-extractable ammonium and nitrate; mg N kg^{-1} soil) ± SEM at three depths before and after grazing.

		Depth (cm)					
		24 June 2015			1 September 2015		
	Species	0-10	10-30	30-60	0-10	10-30	30-60
Total N	BFT	1.6 (0.1) [a]	1.4 (0.1) [a]	0.9 (0.1) [b]	1.1 (0.1)	1.3 (0.1)	1.0 (0.1)
	CMV	1.7 (0.1) [a]	1.3 (0.1) [ab]	1.0 (0.1) [b]	1.4 (0.1) [a]	1.2 (0.1) [ab]	0.9 (0.1) [b]
	MB	1.7 (0.1) [a]	1.3 (0.1) [ab]	1.0 (0.1) [b]	1.3 (0.1)	1.2 (0.1)	0.9 (0.1)
	SB	1.9 (0.1) [a]	1.1 (0.1) [b]	0.9 (0.1) [b]	1.2 (0.1)	1.2 (0.1)	0.8 (0.1)
NH$_4^+$	BFT	1.20 (0.18) [a]	0.36 (0.09) [B b]	0.73 (0.10) [a]	1.09 (0.21)	0.97 (0.14)	1.25 (0.14)
	CMV	0.83 (0.18)	1.36 (0.09) [A]	0.91 (0.10)	1.56 (0.21)	1.30 (0.14)	1.27 (0.14)
	MB	1.70 (0.18) [a]	0.81 (0.09) [A b]	0.67 (0.10) [b]	2.09 (0.21) [a]	1.21 (0.14) [ab]	1.06 (0.14) [b]
	SB	1.84 (0.18) [a]	0.80 (0.09) [A b]	1.09 (0.10) [a]	2.04 (0.21)	1.22 (0.14)	1.32 (0.14)
NO$_3^-$	BFT	10.13 (1.73) [a]	3.37 (0.66) [AB b]	6.93 (0.93) [a]	22.94 (2.63) [A]	19.77 (2.23) [A]	25.89 (2.78) [A]
	CMV	12.58 (1.73) [a]	8.43 (0.66) [A ab]	7.00 (0.93) [b]	19.24 (2.63) [A]	16.18 (2.23) [A]	15.80 (2.78) [A]
	MB	12.81 (1.73) [a]	4.19 (0.66) [AB b]	6.92 (0.93) [ab]	22.74 (2.63) [A ab]	15.98 (2.23) [A b]	31.45 (2.78) [A a]
	SB	4.98 (1.73) [a]	1.67 (0.66) [B b]	3.11 (0.93) [a]	5.27 (2.63) [B]	5.23 (2.23) [B]	6.42 (2.78) [B]
Organic N	BFT	1.6 (0.1) [a]	1.4 (0.1) [a]	0.9 (0.1) [b]	1.1 (0.1)	1.3 (0.1)	1.0 (0.1)
	CMV	1.7 (0.1) [a]	1.3 (0.1) [ab]	0.9 (0.1) [b]	1.3 (0.1) [a]	1.2 (0.1) [ab]	0.8 (0.1) [b]
	MB	1.7 (0.1) [a]	1.3 (0.1) [ab]	1.0 (0.1) [b]	1.2 (0.1)	1.2 (0.1)	0.9 (0.1)
	SB	1.8 (0.1) [a]	1.1 (0.1) [b]	0.9 (0.1) [b]	1.1 (0.1)	1.2 (0.1)	0.8 (0.1)

[A-B] LSmeans in columns with different uppercases letters differ (P<0.05). [a-b] LSmeans in rows with different lowercases letters differ (P<0.05). LSmeans based on 5 spatial replications.

TABLE 2-2 Soil carbon (C) concentrations (g C kg^{-1} soil) ± SEM at three depths before and after grazing.

		Depth (cm)					
		24 June 2015			1 September 2015		
Soil Properties	Species	0-10	10-30	30-60	0-10	10-30	30-60
Total C	BFT	16.5 (0.8) [a]	9.1 (0.7) [B b]	16.3 (1.1) [a]	17.0 (0.8) [a]	11.5 (0.7) [b]	11.0 (1.1) [b]
	CMV	18.0 (0.8) [a]	8.6 (0.7) [B b]	16.9 (1.1) [a]	17.4 (0.8) [a]	12.1 (0.7) [b]	16.8 (1.1) [a]
	MB	15.8 (0.8) [a]	12.4 (0.7) [A b]	15.5 (1.1) [ab]	17.3 (0.8) [a]	13.1 (0.7) [b]	17.6 (1.1) [a]
	SB	16.5 (0.8) [a]	10.6 (0.7) [AB b]	12.2 (1.1) [ab]	16.8 (0.8) [a]	11.8 (0.7) [b]	15.1 (1.1) [ab]
Inorganic C	BFT	1.5 (0.3) [b]	1.6 (0.4) [b]	7.0 (0.6) [a]	0.9 (0.3) [c]	1.8 (0.4) [b]	4.8 (0.6) [B a]
	CMV	1.6 (0.3) [b]	1.1 (0.4) [b]	7.8 (0.6) [a]	1.2 (0.3) [c]	2.2 (0.4) [b]	9.1 (0.6) [A a]
	MB	1.7 (0.3) [c]	2.6 (0.4) [b]	7.0 (0.6) [a]	1.3 (0.3) [c]	2.2 (0.4) [b]	9.2 (0.6) [A a]
	SB	1.5 (0.3) [b]	1.4 (0.4) [b]	4.4 (0.6) [a]	1.3 (0.3) [c]	2.2 (0.4) [b]	9.2 (0.6) [AB a]
Organic C	BFT	15.0 (0.6) [a]	7.5 (0.5) [B b]	9.3 (0.6) [b]	16.1 (0.6) [a]	9.7 (0.5) [b]	6.2 (0.6) [c]
	CMV	16.4 (0.6) [a]	7.4 (0.5) [B b]	9.1 (0.6) [b]	16.2 (0.6) [a]	10.0 (0.5) [b]	7.7 (0.6) [b]
	MB	14.2 (0.6) [a]	9.8 (0.5) [A b]	8.6 (0.6) [b]	16.0 (0.6) [a]	10.1 (0.5) [b]	8.4 (0.6) [b]
	SB	14.9 (0.6) [a]	9.2 (0.5) [AB b]	7.9 (0.6) [b]	15.6 (0.6) [a]	9.6 (0.5) [b]	8.5 (0.6) [b]

[A-B] LSmeans in columns with different uppercase letters differ (P<0.05). [a-c] LSmeans in rows with different lowercases letters differ (P<0.05).
LSmeans based on 5 spatial replications.

TABLE 2-3 Pre- and post-grazing pasture dry matter (kg ha^{-1}) ± SEM obtained using a rising plate meter and their difference.

Species	Pre-grazing	Post-grazing	Forage Disappearance
BFT	4687 (132) [b]	2719 (98) [a]	1998 (139) [b]
CMV	5345 (132) [a]	2652 (98) [a]	2733 (139) [a]
MB	3060 (132) [c]	1396 (98) [b]	1664 (136) [b]

[a-c] LSmeans in columns with different letters differ (P<0.05).
LSmeans based on 5 spatial replications.

TABLE 2-4 Pasture nutritional value (g kg^{-1} DM) $\pm$ SEM, mean of weekly samples.

	BFT	CMV	MB
CP[1]	225.1 (4.1) [b]	258.7 (4.1) [a]	193.2 (4.2) [c]
aNDF[2]	328.5 (5.8) [b]	247.7 (5.7) [c]	543.1 (5.8) [a]
ADF[3]	274.1 (4.5) [b]	223.0 (4.4) [c]	325.0 (4.5) [a]
ADL[4]	67.0 (0.9) [a]	58.9 (0.9) [b]	32.3 (0.9) [c]
NFC[5]	381.5 (3.0) [b]	410.6 (3.1) [a]	166.1 (3.1) [c]
Fat	13.8 (0.5) [b]	14.3 (0.5) [b]	24.5 (0.5) [a]
TDN[6]	713.0 (5.1) [b]	771.2 (5.0) [a]	655.0 (5.1) [c]
DDM[7]	675.5 (3.5) [b]	715.3 (3.4) [a]	635.9 (3.5) [c]
NDFD[8]	412.9 (13.4) [b]	660.1 (13.4) [a]	680.4 (13.5) [a]
Ash	62.2 (1.6) [c]	78.0 (1.6) [b]	92.7 (1.6) [a]

[a-c] LSmeans in rows with different letters differ (P<0.05). LSmeans based on 5 spatial replications and 6 weeks within the grazing season.

[1]CP= crude protein.
[2]aNDF= amylase-treated neutral-detergent fiber.
[3]ADF= acid-detergent fiber.
[4]ADL= acid-detergent lignin.
[5]NFC= non-fibrous carbohydrates.
[6]TDN= total digestible nutrients.
[7]DDM= digestible dry matter.
[8]NDFD= neutral-detergent fiber digestibility.

TABLE 2-5 Values of $\delta^{15}N$ for shoot obtained by the ^{15}N natural abundance method, along with the proportion of legume nitrogen derived from N_2 fixation (P_{fix}) and total N_2 fixed pre- and post-grazing. Isotopic fractionation of the same legumes grown from seed in sand culture without external N was used to calculate P_{fix}.

Species	$\delta^{15}N$ (‰)	P_{fix} (%)	N_2 fixed (kg ha^{-1})
		17 June 2015	
BFT	9.06 ^{c}	49.5	127
CMV	10.11 ^{c}	51.3	142
MB	34.02 ^{a}		
SB	22.17 ^{b}		
S.E.M.	2.88		
		10 August 2015	
BFT	3.34 ^{b}	58.7	67
CMV	7.40 ^{ab}	43.8	59
MB	16.29 ^{a}		
SB	14.21 ^{ab}		
S.E.M.	2.88		

[a-c] LSmeans in columns with different letters differ (P<0.05). LSmeans based on 5 spatial replications.
$\delta^{15}N$ = ^{15}N Natural Abundance,
P_{fix} = Proportion of BFT and CMV derived from N_2 fixation.

TABLE 2-6 The ^{15}N natural abundance method was used to determine δ^{15}N, proportion

and amount of N in feces that was derived from N_2 fixation.

Species	Total N (g kg^{-1})	δ^{15}N (‰)	CT (g kg^{-1})
		24 June 2015	
BFT	23.75	21.33	23.3
CMV	25.97	27.10	4.1
MB	14.63	35.14	8.7

TABLE 2-7 Dry matter intake (DMI), daily enteric methane (CH_4) emissions, and CH_4 as a function of DMI.

Diets	DMI[1], kg head^{-1} d^{-1}	CH_4[2], g d^{-1}	CH_4[3], g kg^{-1} DMI
BFT	17.1 [b]	154.7 [b]	9.05 [b]
CMV	23.3 [a]	141.0 [b]	6.05 [b]
MB	14.4 [b]	225.8 [a]	15.68 [a]
S.E.M.	2.5	10.8	0.8

[a-b] LSmeans in columns with different letters differ ($P<0.05$). LSmeans based on 5 spatial replications and 5 weeks within the grazing season.

[1] DM intake (kg head^{-1} d^{-1}).

[2] Daily gross methane emissions (g head^{-1} day^{-1}).

[3] Enteric methane per unit of DM intake (g kg^{-1}).

FIGURES
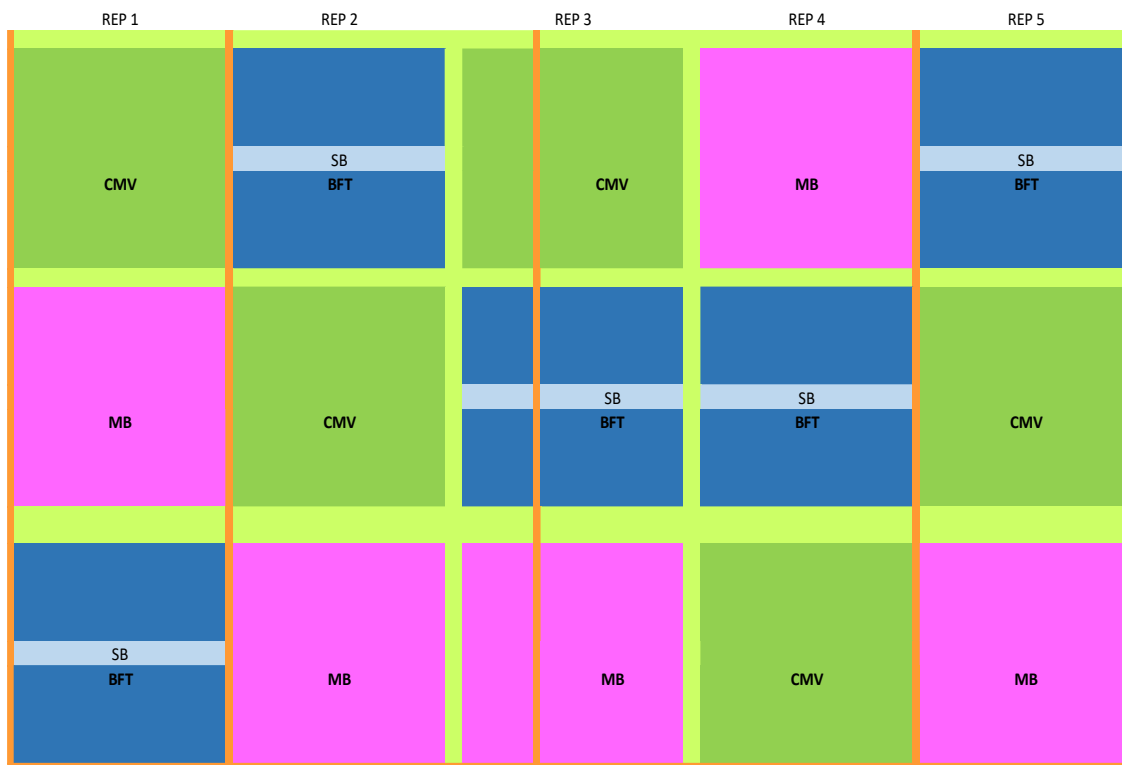
FIGURE 2-1 Pastures plots design: birdsfoot trefoil (BFT), cicer milkvetch (CMV), meadow bromegrass (MB) and small burnet (SB) paddocks randomly distributed across five spatial replications. Each heifer-pasture combination was an experimental unit.
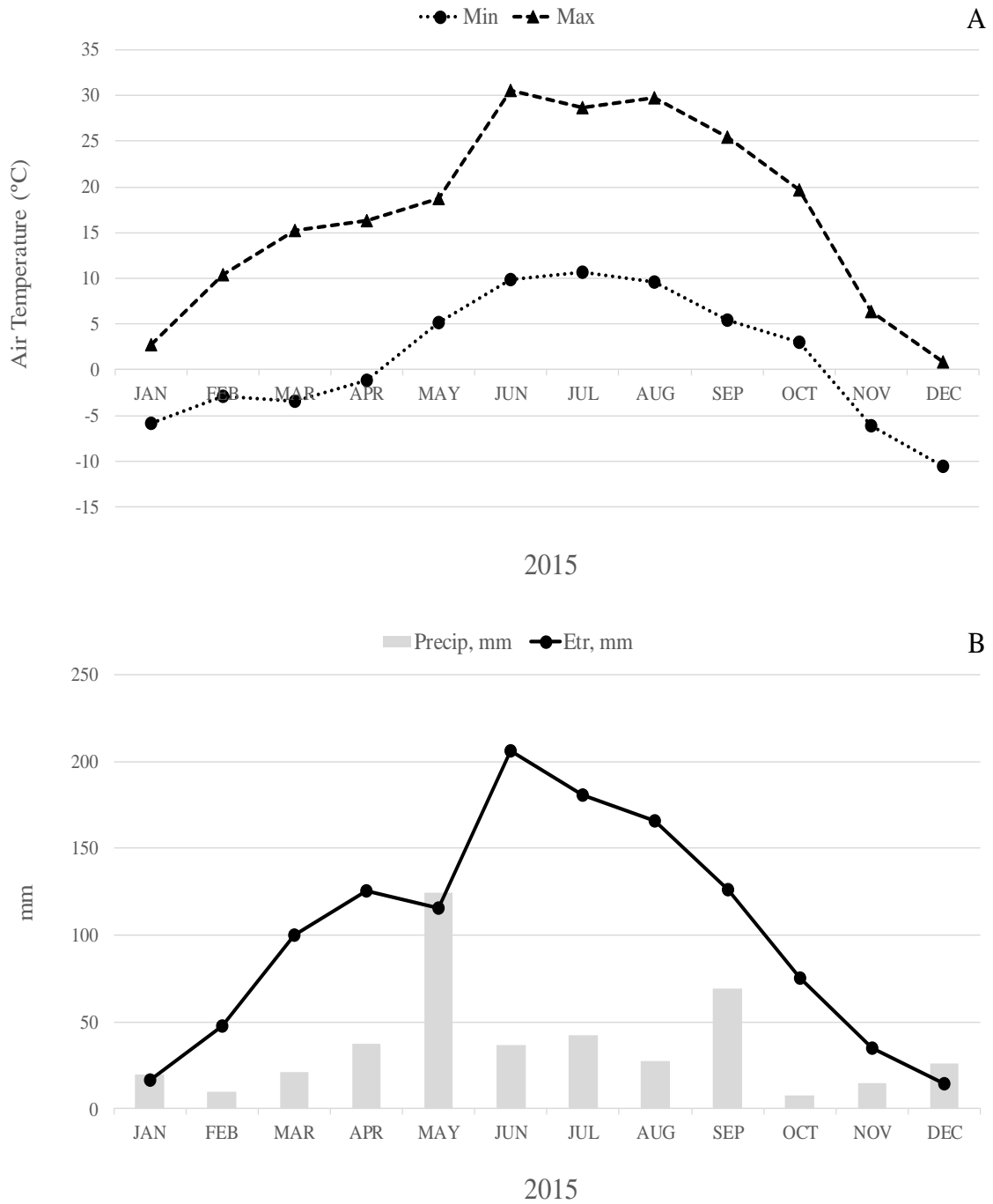
FIGURE 2-2 (A) Average monthly minimum and maximum air temperatures, and (B) monthly evapotranspiration demand (line) and total monthly precipitation (columns) for 2015 at Lewiston, UT.
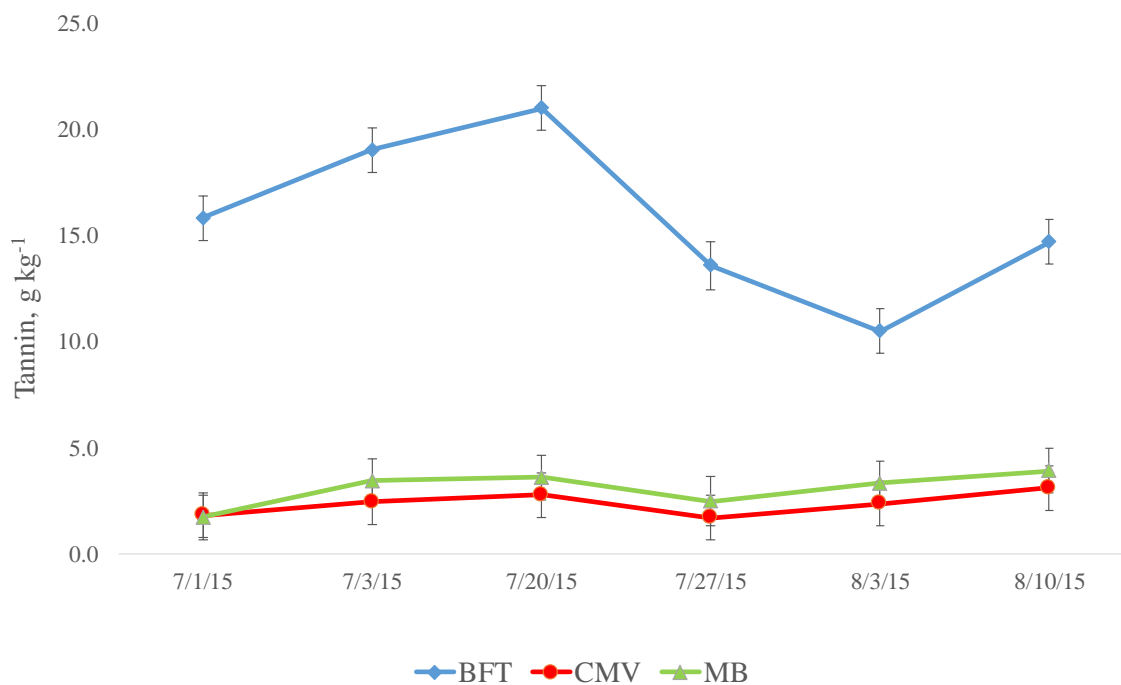
FIGURE 2-3 Tannin concentrations (g kg^{-1}) in shoots of BFT, CMV and MB during the growing season. LSmeans were based on 5 spatial replications (blocks) and 6 weeks within study season. Error bars represent ± SEM.
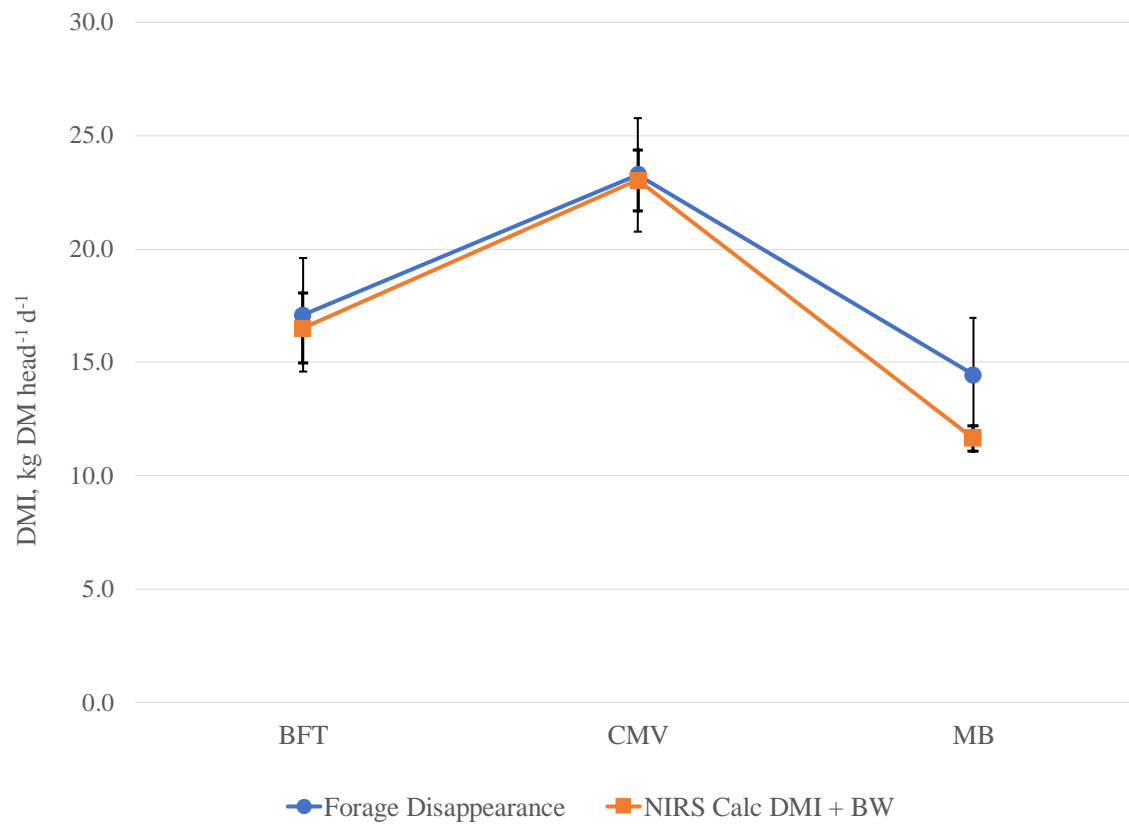
FIGURE 2-4 Two approaches to determination of pasture DMI.

CHAPTER III

FERMENTATION KINETICS AND *IN VITRO* DIGESTIBILITY OF MOUNTAIN

WEST IRRIGATED FORAGE HAYS AND THEIR ISOLATED FIBER

ABSTRACT

The aim of this study was to determine and compare *in vitro* ruminal degradability and gas production kinetics of whole plant and isolated fiber from two condensed tannin-containing legumes, birdsfoot trefoil (*Lotus corniculatus* L.; BFT) and sainfoin (*Onobrichis viciifolia* Scop.; SF) and two non-tannin legumes, cicer milkvetch (*Astragalus cicer* L.) and alfalfa (*Medicago sativa* L.; ALF) relative to a hydrolysable tannin-containing forb, small burnet (*Sanguisorba minor* Scop.; SB) and a cool season grass, meadow bromegrass (*Bromus biebersteinii* Roem. & Schult; MB) using *in vitro* rumen fermentation. Cicer milkvetch had greater dry matter digestibility (DDM) than ALF and SB; and greater organic matter digestibility (DOM) than other species, likely explained by CMV's greater leaf proportion. Digestible DM and DOM of whole plant material were greater than of isolated NDF, and undigested OM was less for whole plant than for isolated NDF. The DDM and DOM of isolated fiber was similar for ALF and SF, and for BFT and SB, suggesting that residual tannins in fiber did not alter microbial fermentation. Across species, whole plant material produced more gas (Parameter A), reached one-half asymptotic gas production more quickly (Parameter B), reached maximum fermentation rate sooner (T_{Max}), and had greater values of maximum fermentation rate (R_{Max}) than isolated NDF. Parameter C of isolated NDF indicated a

more pronounced lag time than for whole plant material, probably due to slower microbial colonization in the absence of rapidly fermentable substrates. Greater cumulative gas of both whole plant and isolated fiber for MB than for most other species, and greater DDM and DOM for CMV than for most other species suggests that the reduced lignin concentration characteristic of these two species allows more of their cellulose to be digested. Greater time to reach Parameter B for both types of MB material likely could be explained by slower rumen microbe colonization. While tannins in isolated NDF of SB and SF did not reduce the extent of fiber digestion, residual tannins may impact the rate of colonization. Based on this study, the greatest whole plant dry matter intake would be expected for the legume hays, due to their higher fermentation rates at the beginning of the incubation process (R_{Max}; CMV and SF) or shorter half-time to maximum asymptotic gas production (Parameter B; ALF and BFT), resulting in lower total gas production for all legumes, faster rates of passage and reduced rumen fill.

Key Words: hay, legume, fermentation, tannin, lignin, digestion.

1 INTRODUCTION

Perennial legume forages have a demonstrated ability to replace grains in finishing diets for ruminants (MacAdam and Villalba, 2015; Chail et al., 2016). Compared with grasses like meadow brome (*Bromus biebersteinii* Roem. & Schult.; MB), legumes have greater nutritive value, lower neutral detergent fiber (Wen et al., 2002), and retain greater feed quality as they mature (Waghorn and Clark, 2004). In the rumen, the particle size of legumes is reduced more rapidly than for grasses,

increasing digestion rate, reducing rumen retention time, improving voluntary intake (Van Soest, 1994; Wilson, 1994) and, consequently, improving ruminant production. In addition, legumes have the capacity to fix their own nitrogen (N) from atmospheric N_2, reducing the input of chemical N fertilizers to the system, decreasing input costs and reducing negative environmental impacts.

Lignin is associated with reduced fiber digestibility and rate of passage of forage from the rumen (Jung and Allen, 1995) and as a consequence, limits voluntary forage intake (Van Soest, 1994). Legumes and grasses differ in lignin concentration (Hoffman et al., 1993), chemical composition (Jung, 1989) and physical location within plant cell walls (Wilson, 1993). Lignin concentrations are greater in legumes than in grasses at comparable levels of dry matter digestibility (Buxton and Russell, 1988; Minson, 1990; Hoffman et al., 1993), greater in stem than leaf tissue (Akin, 1989; Wilson, 1993) and increase with the physiological maturity of forages (Morrison, 1980). Because lignin is more concentrated in legumes, there is greater potential for lignin to reduce digestion (Wilson et al., 1991). In grasses, lignin concentrations are less but lignin more strongly inhibits cell wall digestibility due to an alternative chemical composition (Jung, 1989), with an overall negative impact on animal performance.

Forage plant secondary compounds such as condensed tannins (CT) may also affect animal health and nutrition. Animals consuming CT-containing legumes like birdsfoot trefoil (*Lotus corniculatus* L.; BFT) and sainfoin (*Onobrychis viciifolia* Scop.; SF) have reduced methane emissions (Waghorn, 2008; Guglielmelli et al., 2011), are not at risk of bloat (Wang et al., 2015), or extensive parasitic infections (Waghorn, 1996; Min

et al., 2003; Hoste et al., 2012). The non-tannin species cicer milkvetch (*Astragalus cicer* L.; CMV) is non-bloating because of its leaf morphology, which slows penetration by rumen microorganism through the epidermis (Lees et al., 1982).

The advantages described above for perennial legumes are achieved without a negative impact on dry matter intakes or fiber digestion (Ramírez-Restrepo and Barry, 2005; Ramírez-Restrepo et al., 2006). Thus, BFT and SF CTs bind excess plant proteins in the rumen but allow protein to be released in the abomasum where it can be digested and essential amino acids absorbed from the intestines, increasing the rumen bypass protein (Mueller-Harvey, 2006; Waghorn, 2008; Koenig and Beauchemin, 2018). The BFT and SF CTs reduce N emissions by shifting N excretions from urine to feces, increasing N retention (Koenig and Beauchemin, 2018; Lagrange and Villalba, 2019; Stewart et al., 2019).

Some studies have reported that concentrations of CT greater than 5% may reduce fiber digestion through formation of a CT-microbial enzyme complex (Barry and Manley, 1986; Bae et al., 1993; Min et al., 2003) inactivating microbial enzymes that participate in the digestion process (Reed, 1995). Interference with microbial attachment to feeds that reduced volatile fatty acid (VFA) concentrations, rumen gas production, and ruminant productivity has been documented (Barry and Duncan, 1984; Barry and Manley, 1986; Min et al., 2003). The hydrolysable tannins (HT) that occur in small burnet (*Sanguisorba minor* Scop.; SB) also reduce protein availability in the rumen (Hervás et al., 2000; Stewart et al., 2018). Hydrolyzable tannins are associated with negative effects on intake and digestibility (Verheyden-Tixier and Duncan, 2000;

Ekambaram et al., 2016), and high concentrations have caused damage to the gastrointestinal track, the kidneys and the liver of ruminants (Reed, 1995).

Because of the importance of forage digestibility to ruminant production, an *in vitro* study of the digestibility of dry matter (DDM) and organic matter (DOM), and fermentation kinetics parameters of six forage species was carried out for 96 h using whole ground material and isolated NDF from six hays. Two condensed tannin-containing legumes, BFT and SF, two tannin-free legumes CMV and alfalfa (*Medicago sativa* L.; ALF), a hydrolysable tannin-containing forb SB and a grass MB were studied. Gas production was assessed using the gas production technique described by Theodorou et al. (1994) in order to estimate the kinetics of forage fermentation, which is a good predictor of forage intake (Menke and Steingass, 1988).

2 MATERIALS AND METHODS

2.1 Substrates

Hay of the six species (BFT cv. Langille; SF cv. Shoshone; CMV cv. Monarch; ALF cv. DKA43-22RR; SB cv. Delar; MB cv. Cache) was harvested in early June 2016 at the Utah State University Cache Junction (UT) Farm (41° 51' N, 112° 0' W; elevation 1356 m). Bales weighing ~600 kg were transported to the Utah State University Animal Science Farm in Wellsville, UT, and stored under cover. Bales were sampled using a hay probe, and ~ 500g of each species was freeze dried and ground to pass the 1-mm screen of a Willey mill (Thomas Scientific, Swedesboro, NJ, USA).

2.2 NDF isolation

Ninety 0.5 g subsamples of ground hay of each species was heat-sealed in preweighted ANKOM F57 filter bags, reweighed, and NDF isolated using an ANKOM[200] Fiber Analyzer (ANKOM Technology, Macedon, NY). Twenty-two bags of a given sample, one ALF check, and one blank bag were processed in each run with 2 L of ANKOM neutral detergent (ND) solution, 20 g sodium sulfite and 4 mL α-amylase. Following 75 minutes of extraction, filter bags were rinsed twice for 5 minutes in the ANKOM A200 in 2 L hot water containing 4 mL α-amylase with agitation, and a third 5-min rinse in hot water with agitation. Following the third rinse, water was pressed from the filter bags and they were soaked for 5 minutes in acetone, then dried in a forced-air oven at 102°C and weighed to determine aNDF concentration. After this, dried fiber in filter bags was rinsed three times with hot water and 100 mL of ethanol (Doane et al., 1997). Residual detergent was removed by soaking overnight at 39°C in a solution of 1:9 v/v t-butanol:1 M $(NH_4)_2SO_4$. The isolated fiber was filtered and rinsed with hot water followed by ethanol and acetone (Doane et al., 1997). After drying, filter bags were opened and isolated fiber ground with a coffee mill.

2.3 Fermentation buffer medium

The buffer medium was prepared from deionized water, micromineral solution, artificial saliva, macromineral solution, resazurin (redox potential indicator) and reducing agent according to Menke and Steingass (1988). All reagents were from Sigma-Aldrich (Milwaukee, WI, USA). The pH of the buffer medium was 8.1 ± 0.4.

2.4 Microbial inoculum

Ruminal fluid was collected from a rumen-fistulated Angus beef cow (Utah State University Institutional Animal Care and Use Committee, Approval # 2717) fed on a medium quality alfalfa hay. Four h after feeding, fluid was squeezed from the mat in the ventral region of the rumen into pre-warmed (39°C) thermal flasks and rapidly transported to the laboratory. Once in the laboratory, rumen fluid was squeezed through four layers of cheesecloth into a CO_2-filled 2 L Erlenmeyer flask, mixed and maintained under CO_2 in a water bath at 39°C (Theodorou et al., 1994; Mauricio et al., 1999). Rumen liquor pH was measured with a pH meter (HI 991002, Hanna Instruments, Woonsocket, RI, USA) and averaged 6.5 ± 0.4.

2.5 In vitro gas production technique

In vitro fermentation kinetics were determined during a 96 h incubation of forage substrates with buffered rumen inoculum using the gas production method of Theodorou et al. (1994), followed by assessment of DDM and DOM for whole plant or isolated NDF. Incubations were conducted in gas-tight culture bottles, enabling gases to accumulate in the head-space (closed system) as the fermentation proceeded. Each 125 mL serum bottle (Wheaton, Boston, USA) contained 0.5 g of forage substrate to which 40 mL of buffer was added. Serum bottles were flushed with CO_2 and sealed with 20 mm butyl rubber stoppers and an aluminum crimp cap. Serum bottles with substrate and buffer medium were stored overnight at 4°C to hydrate plant material. The following morning, while the ruminal fluid was being collected, serum bottles were warmed to

39°C in an incubator, then 20 mL of rumen fluid was injected using an 18-gauge needle

(1:2 v/v, rumen fluid:medium ratio), and displaced gas was allowed to escape prior to

removing the needle. Serum bottles were agitated, and incubated at 39°C (Mauricio et al.,

1999). Blanks contained only buffer and rumen fluid, and each 96-h incubation contained

triplicate samples. Readings of headspace gas pressure were made with a USB-output

pressure transducer (PX409-015GUSBH, Omega Engineering Inc., Stamford, CT, USA).

The transducer was connected to a 23-gauge needle that was inserted through the butyl

rubber stopper to read the gas pressure (Theodorou et al., 1994). After each reading, the

transducer was unplugged from the needle to release accumulated gas (Mauricio et al.,

1999). Contents of serum bottles were swirled to mix and returned to the incubator until

the next reading (Theodorou et al., 1994). Pressure readings were taken in the same order

as bottles were injected with the rumen fluid and the gas-measurement, gas-release

procedure was repeated at 1, 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, 72 and 96 h after initiation.

After 96 h, bottles were placed in a walk-in freezer until they reached 4°C to quickly

attenuate microbial activity. The pH was measured as each bottle was opened.

2.6 Fermentation kinetics curve

Gas pressure values were converted to volume according to Equation 1 (Frutos et

al., 2002), expressed on the basis of substrate organic matter (OM) and corrected for gas

released from blanks at each measurement interval. Fermentation kinetics parameters

were derived from cumulative gas production profiles for each hay species using the

Groot et al. (1996) single phase model (Equation 2),

(1)   Head-space gas volume (ml) = 5.3407 * gas pressure reading (psi)

(2)   G = A/(1 + (B^c/t^c))

where *G* (mL/g OM) denotes gas produced per gram of OM at time *t* after the beginning

of the incubation; *A* (mL/g OM) represents asymptotic gas production; *B* (h) is the time

after starting incubation at which half of the asymptotic gas volume has been formed; and

C is a constant describing the sharpness of the switching characteristics of the curve. As

the value of *C* increases, the curve becomes sigmoidal with increasing slope. These

fermentation kinetics were used to calculate the maximum rate of gas production (R_{Max})

and the time at which R_{Max} occurred (T_{Max}) according to the following equations (Bauer

et al., 2001):

(3) R_{Max} (mL h^{-1}) = (A*B^C*C*$T_{Max}^{(-C-1)}$) / ((1 + B^C*T_{Max}^{-C} $)^2$)

(4) T_{Max}(h) = B * (((C-1)/(C+1))$^{1/C}$)

R_{Max} "is reached when the microbial population no longer limits fermentation and

digestion is not hampered by chemical or structural barriers" (Groot et al., 1996).

2.7 Substrate disappearance

The moisture concentration of each whole forage and isolated fiber substrate was

determined by drying at 105°C for 48 h. After fermentation was completed, undigested

residues were filtered through 50 µm-porosity Dacron bags, washed with deionized

water, and dried at 60°C for 48 h. Digestible DM (DDM) was calculated by subtracting

undigested residue DM from substrate DM. Ash was determined by incinerating

fermentation residues at 550°C for 6 h, and substrate OM was calculated by subtracting

ash from substrate DM. Digestible OM (DOM) was calculated as DDM minus ash.

Undigested OM was undigested residue DM minus ash. Lastly, the efficiency of

fermentation was estimated as the partitioning factor (PF), which relates DOM to total

gas production at 96 h (OM disappearance/total gas production; Blümmel et al., 1997).

2.8 Forage chemical analysis

Crude protein (CP), aNDF, NDF digestibility (NDFD), acid detergent fiber

(ADF), NFC (non-fibrous carbohydrates), ash, acid detergent lignin (ADL), fat and total

digestible nutrients (TDN) in hay were determined by near infrared spectroscopy (NIRS).

In vitro true DM digestibility (IVTDMD) of near-infrared reflectance spectroscopy

(NIRS) calibration samples was determined by incubating samples in buffered rumen

fluid for 48 h followed by refluxing of indigestible residues in neutral detergent solution

(Goering and Van Soest, 1970; Peters, 2013). The acid detergent fiber (ADF), CP,

amylase-treated NDF, acid detergent lignin (ADL), and ash of NIRS calibration samples

were made according to AOAC International (2012) methods 973.18, 984.13, 2002.04,

973.18, and 942.05, respectively. Nonfibrous carbohydrate (NFC) concentration was

calculated similarly to NRC (2001) as 1000 - [(NDF-20) + CP + 25 + ash], which

assumes concentrations of 20 and 25 g kg^{-1} for neutral detergent insoluble CP (Peters,

2013) and fat, respectively (NIRS Forage and Feed Testing Consortium, Hillsboro, WI).

Neutral detergent fiber digestibility (NDFD, as a proportion of NDF) was calculated from

NDF and IVTDMD concentrations (Peters, 2013). TDN was calculated from NFC, CP,

fat, aNDF, and NDFD48 using formulas of Undersander and Moore (2002).

These forages were also analyzed for total N (AOAC, 1990; method 990.03)

which was multiplied by 6.25 to estimate CP, NDF (Van Soest et al., 1991), ADF

(AOAC, 1990; method 973.18), and total non-structural carbohydrates (ethanol: (DuBois

et al., 1956), and starch: (Hall, 2009) at Utah State University Analytical Laboratories

(USUAL) in Logan (Utah). Total condensed tannins were determined in triplicate

according to the butanol-HCl-acetone spectrophotometric assay (Grabber et al., 2013)

using reference CT standards isolated from SF and BFT (Hagerman, 2011) and the HT

concentration of SB was determined using the method of Hartzfeld et al. (2002).

2.9 Experimental design and statistical analysis

The experimental design was a completely random block design with four runs

(spatial replication), six hays species (ALF, BFT, CMV, SF, MB and SB) and two type of

substrate (whole plant and isolated fiber) as treatments. Triplicates of each treatment

along with a control (ALF) and a blank were included in each run. All kinetics

parameters were estimated using PROC NLIN, and compared using PROC GLIMMIX in

SAS/STAT 14.3 (SAS Inst., Inc. Cary, NC; Version 9.4 for Windows) with A=200, B=20

and C=1, as initial values. The estimated apparent DDM and DOM, *in vitro* fermentation

kinetics parameters (A, B, and C), R_{Max}, T_{Max}, and partitioning factor (PF) were analyzed

using a mixed model in which run was the random factor, and species and material (whole or isolated fiber) with their interaction were fixed effects. A heterogeneous compound-symmetry (CSH) covariance structure was included to account for correlations of the measurements of the two materials for the same species. Parameter B used lognormal distribution, and parameters A and C, DDM, DOM, T_{Max}, R_{Max} and PF used normal distribution with heterogeneous variance by material. Least squares means (LSMeans) were compared pairwise using Tukey's multiple comparison test when F-ratios were significant ($P<0.05$) and reported along with their standard errors (SEM). A tendency was considered when $0.10 > P > 0.05$. Homoscedasticity of variance and normal distribution were checked using studentized residuals and no apparent violations were found.

3 RESULTS

3.1 Chemical composition of substrates

The nutritive value of the six hays determined by NIRS is reported in Table 3-1, and their composition as determined by wet chemistry, including the CT concentration of BFT and SF hays and the HT concentration of SB hay, are shown in Table 3-2. Data from NIRS and wet chemistry for CP, ADF and NDF were correlated ($P=0.0001$, 0.0058 and 0.0725, respectively). The four legumes and the non-legume forb were similar to one another in CP, NDF and NFC concentrations, while for the grass, NDF was greater and CP was less than for the legumes and forb.

3.2 Digestibility of dry matter and organic matter, and undigested organic matter

After 96 h of incubation, DDM and DOM of whole plant material (Table 3-3) were greater that of isolated NDF ($P<0.001$), and undigested OM was less for whole plant than for isolated NDF ($P<0.001$). The DDM of whole CMV was greater than for whole ALF and SB ($P<0.001$), the DOM of whole CMV was greater than for whole plant material of all other forages, and whole CMV had less undigested OM than all other forages. For isolated NDF, DDM and DOM were greatest for CMV and MB, and greater for BFT and SB than for ALF and SF. The isolated NDF of CMV had the least undigested OM, but was not different from MB.

3.3 Fermentation kinetics parameters

After 96 h of incubation, the pH ranged from 6.2-6.9 for all fermentation solutions, indicating that fiber digestion was not limited by pH and optimal conditions for cellulolytic bacterial activity were maintained during the fermentation process.

Cumulative gas production profiles, rate of gas production curves and parameters describing the cumulative gas production for each forage are presented in Fig. 3-1 and Table 3-4, respectively. There were significant differences among the six hay species for asymptotic gas production (Parameter A) of whole plant material ($P<0.001$) and isolated NDF ($P<0.001$). Across species, whole plant material produced more gas than isolated NDF ($P<0.001$). Species also differed in time needed to reach one-half cumulative gas production (Parameter B) for whole plant material ($P<0.001$) or isolated NDF ($P<0.001$).

The cumulative gas curvature characteristic (Parameter C) did not differ among species for isolated NDF ($P>0.05$) or whole plant material ($P>0.05$).

Asymptotic gas production was greater for MB whole plant material than for whole plant material of CMV, BFT, SF and ALF, although both MB and the legumes did not differ from SB. For isolated fiber, Parameter A was greater for MB than for forages other than CMV, and greater for CMV than for SF and ALF, but CMV did not differ from BFT and the forb SB. Time to one-half asymptotic gas production (Parameter B) was greater for whole plant material of MB than for all species other than whole plant SB. For isolated fiber, Parameter B was greatest for MB; and greater for SB and SF than BFT and CMV; SB and SF did not differ from ALF. Parameter C of isolated NDF indicated a more pronounced lag time and greater slope than whole plant Parameter C ($P<0.05$).

Maximum fermentation rates (T_{Max}) were reached faster for whole plant material than isolated NDF ($P<0.001$), and the maximum fermentation rate (R_{Max}) reached greater values for whole plant material than isolated NDF ($P<0.001$). No differences in T_{Max} were found among species for whole plant material; however, for isolated NDF, less time was needed for ALF, BFT and CMV to reach R_{Max} than for MB, while MB did not differ from SF and SB. For whole plant material, BFT had the greatest R_{Max} but did not differ from ALF and SB, while the R_{Max} of MB was less than for ALF and BFT. For isolated NDF, the R_{Max} of CMV was greatest, followed by BFT, which did not differ from SB, and was least for ALF, MB, and SF. There were no differences among species for

fermentation efficiency (PF) of whole plant ($P>0.05$) or isolated NDF OM disappearance ($P>0.05$).

4 DISCUSSION

4.1 Digestibility of dry matter and organic matter, and undigested organic matter

In the current study, whole plant DDM of CMV was greater than for ALF and SB, and whole plant DOM was greater for CMV than any other forage. The isolated NDF DDM and DOM of both CMV and MB were the greatest among all of the hays assessed. This aligns with results from a study conducted in Minnesota where digestibility of CMV was greater than of other legumes such as ALF, BFT and SF (McGraw and Marten, 1986). The leaf-to-stem ratio in ALF, BFT and SF ranged from 0.36 to 0.41, while the leaf-to-stem ratio in CMV was 0.72. Stems of CMV are viney, and CMV is non-bloating because its leaf structure which slows microbial access to cell contents (Lees et al., 1982). In contrast, the other legumes all have more upright stems than CMV, which lead to greater amounts of undigested OM following fermentation. Several researchers (Jung, 1989; Jung et al., 1993; Guglielmelli et al., 2011) have observed greater DOM in lower ADL forages such as CMV and MB, but in the current study there was no correlation between ADL and DOM of whole plant material or isolated NDF.

In the present study, DOM did not differ among ALF, BFT and SF hays harvested at the early flowering stage, similar to results reported by Kraiem et al. (1990). While a minimal amount of CP is needed for microbial colonization of forages in the rumen (Guglielmelli et al., 2011) there was no correlation of CP contents and digestibility of

whole plant material in the current study. In contrast with results reported by Calabrò et al. (2001), Kaplan (2011) and Han et al. (2013) whole plant NDF, ADF and ADL concentrations in this study were not correlated with *in vitro* whole plant DOM.

Condensed tannins can affect rumen digestion at concentrations above 50 g kg^{-1} (Wang et al., 2015) but concentrations of CT in this study for BFT and SF were 7.5 and 30 g kg^{-1} DM, respectively, which represent values below that threshold. Whole plant DDM did not differ between the tannin-containing forages BFT, SF and SB and non-tanniferous ALF. The DOM of isolated NDF from ALF and SF did not differ and values were less than for the rest of the assayed species, suggesting that tannin residuals in BFT, SF and SB in the isolated fiber were not significant impediments to fiber digestion by rumen microbes. Similar results were reported for Aufrère et al. (2008) and Guglielmelli et al. (2011) where the tannin content of SF hay did not alter microbial fermentation relative to non-tanniferous forages. Field drying of forages before baling reduces extractable forage CT, possibly limiting their biological activity compared with tannins in fresh forage (Wang et al., 2015).

The forb SB had lower values of whole plant DDM and DOM than CMV, despite showing less contents of fiber and greater concentrations of TNC than the four legumes. These results may be explained by the presence of hydrolysables tannins (HT), which possess antimicrobial properties (Ekambaram et al., 2016). Nevetheless, the DDM and DOM of ALF and SB did not differ, altough the isolated fiber of SB had greater DDM and DOM values than that of ALF, suggesting that cellulose in SB was more digestible than cellulose in ALF.

Smith (1964) demonstrated that the crude fiber content of BFT increases less with advancing maturity than that of ALF, increasing its relative digestibility. Several other studies have noted differences in the ratio of cell solubles to cell wall contents between BFT and ALF, suggesting that fiber of ALF is more lignified (Hoffman et al., 1993), since ADL concentration is known to be negatively correlated with fiber digestibility (Jung, 1989). Further, BFT produces finer and less upright stems. The isolated fiber of BFT had greater DDM and DOM than that of ALF. The ADL concentrations of BFT and ALF were similar, but the deposition or chemical nature of the lignin in these two species may differ, leading to greater reductions in the extent of fiber digestion in ALF.

4.2 Fermentation kinetics parameters

Asymptotic gas production (Parameter A) of whole plant material was greater for the grass (MB), than for all four legumes, but it did not differ from SB. Parameter A of isolated fiber was greater for MB than for all species other than CMV and least for ALF. Parameter A is related to the extent of digestion, and for isolated fiber, Parameter A of the 96-h fermentation is essentially a measure of the extent of cellulose digestion, since cell contents and neutral detergent-soluble cell wall constituents were rigorously removed from isolated NDF before fermentation began. Parameter A of the isolated fiber of MB and CMV did not differ, and the DDM and DOM of the isolated fiber of MB and CMV were greater than that of the other four species.

The fact that MB – along with CMV – fermentation produced the most cumulative gas of both whole plant and isolated fiber, with greater DDM and DOM than

in other species, suggests that the reduced lignin concentration characteristic of these two species allows for greater amounts of cellulose to be digested.

The main products of cellulose digestion in the rumen are carbon dioxide (CO_2), methane (CH_4) and short-chain (volatile) fatty acids (VFA), and their production has been positively related to OM fermentation (Calabrò et al., 2001). Parameter A for whole plant material was not correlated across functional groups (grass, legume, forb) with whole plant DOM in the current study, but there was a significant correlation of Parameter A of isolated NDF with the DOM of isolated NDF (P=0.05).

Time to reach one-half of the asymptotic gas production (Parameter B) was the greatest for MB amd SB (whole plant material), and for MB (isolated NDF). This means that the microbial colonization of MB occurred at lower rates than for other species, probably because fiber cells in grasses are longer even though they are less lignified than fiber cells in legumes; microbes can typically digest fiber cells more readily from the inside (lumen), where lignin is least dense (Wilson and Mertens, 1995). Thus, ALF and BFT reached half time to asymptotic gas production more rapidly than the grass.

The time to maximum rate of gas production, T_{Max}, is related to Parameter B. The T_{Max} of whole plant material did not differ among species but the T_{Max} of isolated fiber was greatest for MB and least for ALF, BFT and CMV. It is expected that whole plant material of legumes, with greater concentrations of CP and NFC, would support rapid colonization of whole plant material. Nevertheless, isolated fiber did not contain such nutrients, suggesting that the physical and/or chemical nature of ALF, BFT and CMV cell walls were more supportive of microbial colonization than those of MB. Wilson (1993)

notes that a smaller proportion of tissue is lignified in legumes than in grasses, although that proportion of legume tissue is more intensively lignified. This fundamental difference between grasses and legumes would explain slower colonization of isolated NDF of MB than of ALF, BFT and CMV. Residual tannin in isolated NDF of SB and SF (Table 3-2) may explain why the rate of colonization of SB and SF was intermediate to MB and the three legumes ALF, BFT and CMV.

In contrast with Blümmel and Becker (1997), the asymptotic cumulative gas production, maximum fermentation rate, and time to reach one-half asymptotic cumulative gas ("fermentation process") were greater or more rapid or had less lag time for whole forage than for NDF isolates of the six species assayed. A pronounced lag times in gas production was detected for isolated NDF of each fermented substrate, similar to results of Schofield and Pell (1995) and Calabrò et al. (2001), likely due to slower microbial colonization in the absence of rapidly fermentable substrates (Chesson and Forsberg, 1988; Groot et al., 1996).

Based on our findings, the greatest whole plant dry matter intake would be expected for the legume hays, because of their higher fermentation rates at the beginning of the incubation process (R_{Max}; CMV and SF) or shorter half-time to maximum asymptotic gas production (Parameter B; ALF and BFT), along with lower total gas production for all legumes, indicating a reduced extent of digestion, which could result in faster rates of passage and reduced rumen fill (Van Soest, 1994).

5 CONCLUSIONS

Legumes showed greater CP contents than the grass and forb. While the forb SB had less NDF concentration and more TNC than the grass, it also contained HT that were retained in fiber, causing its fermentation kinetics to resemble that of MB more than the legumes.

Sainfoin, along with ALF and BFT, had more undigested fiber than CMV and MB, and CMV and MB fiber had greater asymptotic cumulative gas production. These differences are likely due to lignin or tannins creating impediments to cellulose digestion. Greater gas production early in fermentation (reduced values for Parameter B) along with reduced cumulative gas production (Parameter A) may predict greater voluntary dry matter intake of forages by ruminants, and could be used as a tool of diet selection in order to improve animal performance. According to these values, we would expect the greatest voluntary dry matter intake for ALF and BFT, followed by CMV and SF, with the least voluntary intake of MB and SB.

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TABLES

TABLE 3-1 NIRS prediction of forage nutritive value of hays forages assayed in this study (g kg^{-1} DM and g kg^{-1} NDF just for NDFD).

Species	CP	ADF	aNDF	NDFD	NFC	Ash	ADL	Fat	TDN
Alfalfa	210.8	281.7	347.9	392.3	360.7	75.7	72.8	12.4	704.3
Birdsfoot Trefoil	168.5	313.5	368.8	386.8	401.5	56.2	71.5	11.6	668.1
Cicer milkvetch	221.3	264.6	317.5	496.3	368.7	87.5	66.0	13.5	723.9
Sainfoin	146.9	353.7	420.2	352.9	376.1	51.7	88.4	3.5	622.2
Meadow brome	90.1	449.4	699.2	530.9	152.2	53.4	49.3	12.9	513.1
Small burnet	139.8	300.3	416.6	530.6	376.6	62.0	85.7	19.4	683.2

CP= crude protein; ADF= acid-detergent fiber; aNDF= neutral-detergent fiber; NDFD= NDF digestibility; NFC= non-fibrous carbohydrates; ADL= acid-detergent lignin; TDN= total digestible nutrients.

TABLE 3-2 Chemical composition and tannin content (mean ± SEM) of the hays species used in the *in vitro* fermentation study (g kg^{-1} DM).

| Species | Ankom | | | | | | Tannins | Tannins |
	CP	aNDF	ADF	TNC	Ethanol	Starch	Whole	NDF
Alfalfa	187±2	438±1	306±1	71±4	64±3	6.5±1		
Birdsfoot trefoil	141±1	433±2	315±3	102±1	93±1	9.5±1	7.5±1	0.67 ± 0.03
Cicer milkvetch	197±1	353±1	283±2	73±3	68±3	5.5±1		
Sainfoin	137±0	448±4	357±4	92±1	79±1	13.5±1	30.0±4	1.14 ± 0.05
Meadow brome	81±1	671±2	416±1	85±2	81±2	4.0±0		
Small burnet	117±1	366±2	243±3	136±0	112±1	24.5±1	41.1±1.8	3.6 ± 2.1

CP= crude protein; NDF= neutral-detergent fiber; ADF= acid-detergent fiber; TNC= total nonstructural carbohydrates; Ethanol= ethanol soluble carbohydrates.

TABLE 3-3 Characteristics of unfractionated and isolated NDF (g kg^{-1} DM) of the hays species used in this *in vitro* fermentation study.

Species	DDM	DOM	Undigested OM	NDF DDM	NDF DOM	Undigested NDF OM
Alfalfa	686^b	649^b	149^a	473^c	447^c	252^{ab}
Birdsfoot trefoil	712^{ab}	667^b	141^a	557^b	533^b	227^{ab}
Cicer milkvetch	766^a	739^a	106^b	709^a	685^a	143^d
Sainfoin	706^{ab}	663^b	146^a	483^c	457^c	258^a
Meadow brome	734^{ab}	674^b	139^a	671^a	648^a	169^{cd}
Small burnet	671^b	651^b	148^a	572^b	549^b	206^{bc}
S.E.M.	24	18	8	15	14	17

Means in a column with different superscript letters differ (P<0.05).

TABLE 3-4 Fermentation kinetics parameters of whole plant material and isolated NDF.

| | Kinetic curve parameters | | | | | |
	A (ml g^{-1} OM)	B (h)	C	T_{Max} (h)	R_{Max} (mL h^{-1})	PF (mg mL^{-1})
Whole:						
Alfalfa	180.2[b]	7.5[c]	1.2	1.0	16.9[ab]	3.80
Birdsfoot trefoil	209.1[b]	8.5[c]	1.1	0.1	20.0[a]	3.57
Cicer milkvetch	210.6[b]	12.9[bc]	1.1	1.1	12.6[bc]	3.97
Sainfoin	189.9[b]	10.9[bc]	1.1	0.9	12.5[bc]	3.57
Meadow brome	250.0[a]	30.0[a]	1.0	0.4	8.4[c]	3.71
Small burnet	213.5[ab]	18.1[ab]	0.9	1.0	12.7[abc]	3.74
S.E.M.	12.5	6.2	0.09	0.6	2.5	0.3
Isolated NDF:						
Alfalfa	109.6[d]	18.0[bc]	2.2	11.0[b]	4.1[c]	4.47
Birdsfoot trefoil	131.8[bcd]	17.0[c]	2.2	10.7[b]	5.3[b]	4.15
Cicer milkvetch	153.4[ab]	14.9[d]	2.3	9.9[b]	7.3[a]	4.55
Sainfoin	118.1[cd]	19.3[b]	2.3	12.4[ab]	4.3[c]	4.49
Meadow brome	176.8[a]	26.4[a]	2.0	15.2[a]	4.4[c]	3.90
Small burnet	139.6[bc]	19.8[b]	2.2	12.3[ab]	4.8[bc]	4.27
S.E.M.	8.2	0.9	0.2	1.7	0.2	0.8

A: Asymptotic gas production (mL g OM^{-1}); B: time to half of the asymptote (h); C: Constant determining the sharpness of the curve; T_{Max}: time at which R_{Max} occurs (h); R_{Max}: maximum gas fermentation rate (mL h^{-1}); PF: Partitioning Factor (mg OM disappeared mL gas produced^{-1}). Means in a column, for each type of sample, with different letters differ significantly ($P < 0.05$).

FIGURES
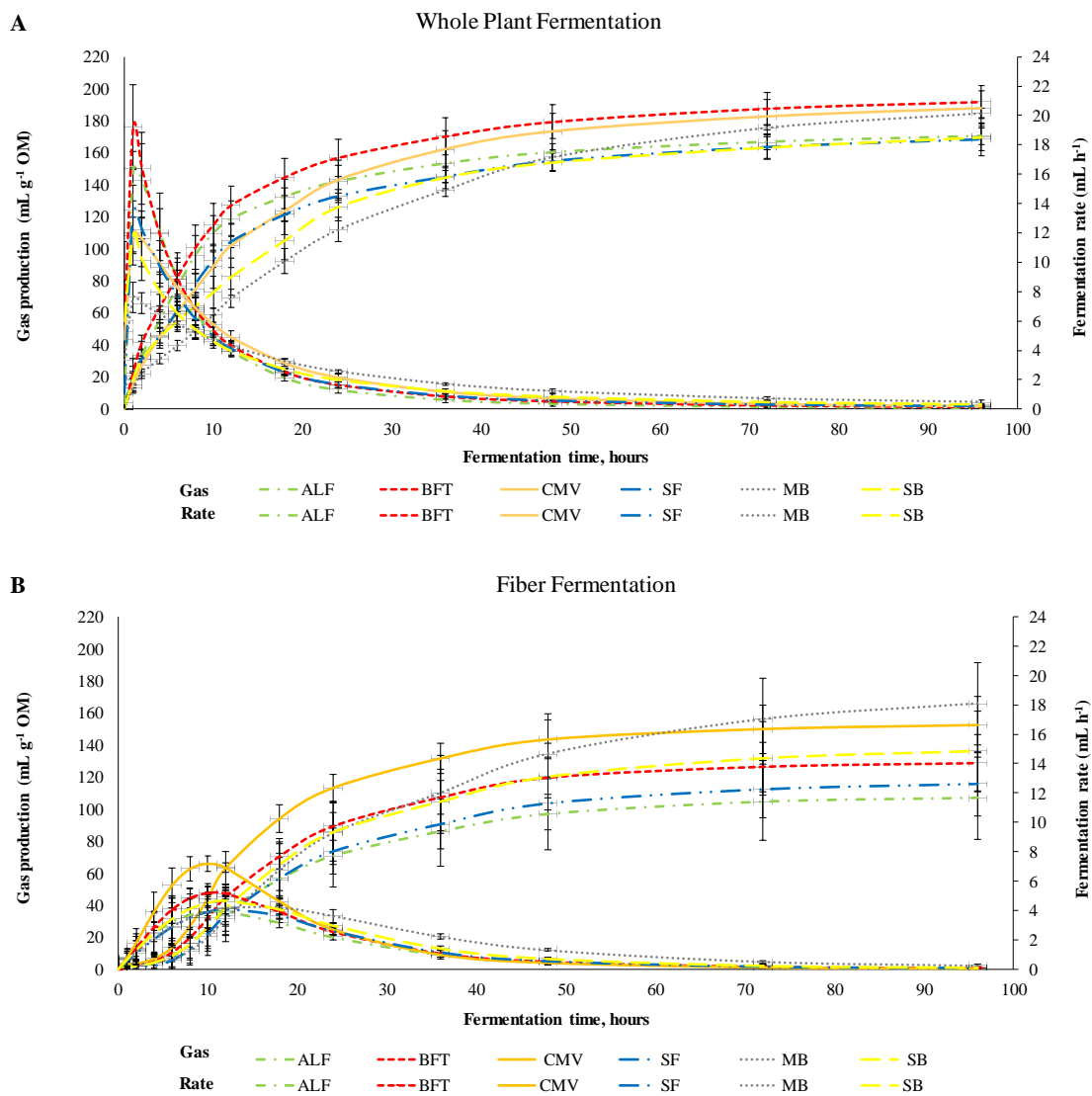
FIGURE 3-1 Cumulative gas production and rate of gas production profiles from whole plant material (A) and isolated NDF (B) of ALF, BFT, CMV, SF, MB and SB.

CHAPTER IV

NITROGEN BALANCES FROM LEGUMES AND NON-FIXING SIMULATED

GRAZING SYSTEMS

ABSTRACT

Nitrogen (N) is the most limiting agricultural nutrient, required for photosynthesis and protein synthesis, necessary for the growth and development of all living organisms on Earth. Legume pasture forages such as birdsfoot trefoil (*Lotus corniculatus* L.; BFT) and cicer milkvetch (*Astragalus cicer* L.) have the unique advantage of fixing their own N from the atmosphere, giving them independence from external chemical fertilizers. Nitrogen fixation is a self-regulating system, capable of using soil N if it is available, which was seen in the current study after the addition of manure. Some forbs such as BFT and small burnet (*Sanguisorba minor* Scop.; SB) synthesize biocomponents such as condensed and hydrolysable tannins, that influence soil N and C cycling while potentially lessening N losses to the environment. A controlled-environment study was conducted to investigate N cycling as well as the contributions of forage species to soil C in pasture systems based on deep-rooted perennial legumes (BFT and CMV), a cool-season grass (meadow bromegrass, *Bromus biebersteinii Roem. & Schult*; MB) and a non-legume forb (SB) that are well-adapted to production in temperate climates under irrigation on alkaline soils. Birdsfoot trefoil produced the greatest amount of herbage DM and accumulated more total herbage N than other species, and fixed more N during the study than CMV. Meadow bromegrass accumulated more root mass and greater root length and

surface densities than other species, but SB root mass did not differ statistically from MB, and SB had more total root length and root surface area by the end of the study than other species. The non-tannin legume CMV produced less herbage DM than the tannin-containing legume BFT and accumulated the greatest soil NO_3^- by the end of the study. Meadow brome and SB invested more C and N in their root systems than the N-fixing legumes. Nitrogen balances revealed an approximate 2-3-fold return on the initial investment of soil organic matter over the simulated three years of grazing for MB and SB, and negligible N leaching, even though the legumes systems gained N from both manure and N fixation. These perennial forage systems all appear to be sustainable, but some produced more herbage DM than others.

Keywords: nitrogen, carbon, legumes, grass, tannins, sustainability.

1 INTRODUCTION

The US Intermountain West is a semi-arid region dominated by low organic matter (OM) calcareous soils (Bui et al., 1990). Some "non-traditional" pasture forage species like birdsfoot trefoil (BFT), cicer milkvetch (CMV), and small burnet (SB) are well-adapted to western U.S. climatic and edaphic conditions (Sheaffer et al., 1993; Ogle, 2002; Ogle et al., 2012). The legumes BFT and CMV have a sustainable ability to fix dinitrogen (N_2) from the atmosphere. Birdsfoot trefoil contains condensed tannins (CT) while the leaf structure of CMV slows digestion, so both legumes are nonbloating (Min et al., 2003; Waghorn, 2008; Wang et al., 2015). Like CT, hydrolysable tannins (HT) in SB inhibit microbial processes related to nitrogen (N) and carbon (C) cycling in soil

(Smolander et al., 2012) but HT can be toxic for ruminants when they are a significant part of the diet (Hervás et al., 2000). Thus, the incorporation of these non-traditional forage species in pasture systems addresses the increasing global concern of N losses to groundwater or the atmosphere from agricultural systems. Several workers have used N cycling to study such N losses (Garrett et al., 1992; Jarvis, 1993; Ledgard et al., 1999); however, those studies were conducted on boreal forest soils or with shallow-rooted pasture plant species such as perennial ryegrass and white clover, and few included a detailed N budget.

In agricultural soils, N is particularly important because it is required for productive soils, plants and ruminant animals, but its loss to the environment is undesirable. Soil N deficiency can limit plant yield and quality (protein concentration) of crops. Nitrogen comprises about 79% of atmospheric gases, and 99% of atmospheric N is in the form of N_2, which is inert and cannot be used directly by most living organisms (Marschner, 2012). Biological nitrogen fixation (BNF) is more sustainable than chemical N fertilizer because it relies on prokaryotic microorganisms that fix atmospheric N_2 using a specialized enzyme complex, nitrogenase, that functions at atmospheric pressure and ambient temperature, and using energy from photosynthesis. In this process, a molecule of N_2 is reduced to two molecules of ammonia (NH_3) and immediately used to form organic compounds that can be metabolized within the plant to amino acids. Hence, BNF in association with legumes is an environmentally benign alternative to chemical N fertilization. Well-nodulated plants may fix in excess of 200 kg of N ha^{-1} $year^{-1}$ (Weaver

and Danso, 1994), and transfer some of this fixed N to associated grasses in mixed pastures (Ledgard and Steele, 1992).

Readily available soil nitrogen is generally considered to be the sum of ammonium (NH_4^+) and nitrate (NO_3^-) in the soil solution and on exchange sites. Ammonium leaches very little because the cation is held by negatively charged soil surfaces and organic matter (OM) comprising a soil's cation exchange capacity, while NO_3^- is repelled by the same charges. In fertilized crops or under manure application, excess soil NO_3^- is an environmental concern because it is readily leached into ground water, streams and lakes by irrigation or precipitation that exceeds crop water use (Pierzynski et al., 2000; Norton, 2008), contributing to eutrophication. Nitrogen mineralization and nitrification are key N transformations that largely determine the availability and mobility of N in soils (Norton 2008; Norton and Stark, 2011). The mineralization of OM results in the formation of NH_4^+, which is then rapidly converted to NO_3^- by the process of nitrification, which accumulates in the soil solution (Norton, 2008). Nitrification is an aerobic process regulated by the availability of NH_4^+, which depends on the C:N in OM and the microbial activity that mineralizes NH_4^+. Plants and soil microbes can use N as either NO_3^- or NH_4^+, but if it is available, the assimilation of NH_4^+ -N costs plants and microbes less metabolic energy than the assimilation and reduction of NO_3^- -N (Schlesinger, 1997).

Nitrogen losses occur by NO_3^- leaching, volatilization of ammonia (NH_3) and by denitrification. Ammonia, nitrous oxide (N_2O) and nitric oxide (NO_x) are the main gaseous products that are responsible for degradation of air quality and contribute to

GHG production. Under grazing, most of the N in urine from cattle is in the form of urea, which is rapidly converted to plant-available N (Whitehead, 1995); the N in feces must be mineralized by soil microbes to become plant-available N (Haynes and Williams, 1993; Menneer et al., 2004). The N in manure, a mixture of urine and feces, is subject to substantial losses via NH_3 volatilization during application or deposition. However, if the soil is well-aerated and near a neutral pH, nitrification will be dominant, leading to high concentrations of soil NO_3^- subject to losses by leaching.

Gases such as N_2O and NO_x are produced in soils following microbial nitrification, (the oxidation of NH_4^+ to NO_3^-) and the subsequent reduction of NO_3^- to NO_x, N_2O and N_2 (Stevens et al., 1997). Reducing N_2O-gas emissions is important because its global warming potential (GWP) is 298 times greater than that of carbon dioxide (CO_2) (Forster et al., 2007). This is because it absorbs energy efficiently, it persists longer in the atmosphere than methane (CH_4), and it contributes to ozone depletion (Schmeer et al., 2014). Azam et al. (2002) have reported that N_2O production can occur simultaneously under nitrification and denitrification within the same soil aggregate where aerobic and anaerobic microsites coexist. Furthermore, soil N sources that can result in N_2O gas production and emission include mineral fertilizer, manure ('hot-spot' effects due to excretion of urine and feces), crop residues (legume crop residues usually decompose faster than residues from non-legume crops), and BNF of atmospheric N_2 by legume crops (Whitehead et al., 1986; Rochette and Janzen, 2005; Schmeer et al., 2014).

In legume systems, N_2O may be emitted from the degradation of root nodules where organic N inside the nodules is mineralized to NH_4^+, followed by nitrification and denitrification that produce N_2O (Itakura et al., 2013). The magnitude of N_2O emissions depends on several factors including soil compaction, soil water content (reduced O_2 concentrations in compacted and poorly drained soils), N source (BNF versus mineral N fertilization), concentrations of soil NH_4^+ and NO_3^-, soil temperature, and other climatic conditions (Rochette et al., 2004). It is well-documented that soil compaction increases the emission of N_2O (van Groenigen et al., 2005). Overall, models of N_2O production show that it mainly depends on how much NO_3^- production and accumulation are in the agricultural system; therefore, the challenge is to minimize the accumulation of NO_3^- in the farming system through improved N use efficiency, which will ultimately reduce GHG emissions and ozone depletion from agriculture and improve environmental health.

Carbon (C) availability plays a key role in controlling N cycling in soils. Limited available organic C can impede biological denitrification (Drury et al., 1991) and NO_3^- leaching through its influence on microbial growth (microbial biomass and denitrifiers). Tannins are a group of C-based plant secondary compounds synthesized by some forage legumes and classified into hydrolysable (HT) and condensed tannins (CT). Tannins precipitate proteins and alkaloids, and differ from plant to plant in molecular weight and subunit composition (Zucker, 1983). The CT in legume species such as birdsfoot trefoil (BFT) may bind to protein in the rumen, increasing rumen bypass protein and in the process increase fecal:urinary N in waste (Waghorn et al., 1994; Barry and McNabb, 1999; Woodward et al., 2009). Partitioning more waste N into feces slows the release and

leaching potential of N in pasture agroecosystems (Waghorn, 2008; Woodward et al., 2009).

Condensed and HT have been shown to regulate N and C cycling in soil (Smolander et al., 2012) by inhibiting soil microbial mineralization of feces and nitrification (Adamczyk et al., 2013), thereby increasing N immobilization into soil organic matter (SOM), increasing soil C storage and reducing nutrient losses to the environment. Consequently, pastures with significant tannin-containing legumes reduce the load of GHG per unit of forage produced (reducing the C footprint), increase C sequestration, enhance soil quality and nutrient cycling, increase nutrient storage and overall increase the sustainability of agriculture systems by reducing N and C losses.

The distribution of N deposition onto grazed pasture soils is inherently variable, and this variability was apparent in the NH_4^+ and NO_3^- values of soil samples taken from pastures before and after grazing in our 2015 field study. Therefore, a controlled environment assessment of soil N cycling using the same perennial forages under simulated grazing was carried out at the USU Research Greenhouses. In this study, N balances were developed under simulated grazing, where inputs and outputs could be compared for CT and non-CT legumes, an HT-containing forb and a grass. Manure collected from cows fed each of the same four plant species was applied in the same volumes to the grass, forb and legumes. We hypothesized that N and C losses would be less under the tannin- containing legume and forb because soil microbial processes (mineralization and nitrification) would be inhibited, reducing NO_3^- leaching and

resulting in greater environmental sustainability through mitigation of agricultural N losses.

2 MATERIALS AND METHODS

2.1 Growth conditions

The study was carried out in 20-cm-dia. x 80-cm-deep polyvinylchloride (PVC) cylinders closed on the bottom with PVC caps and lined with polypropylene film sealed on the bottom to allow removal of excess irrigation water using a suction lysimeter located in the center of the column. Lysimeters were constructed of 1-m-long 12.7-mm-o.d. schedule 40 polyvinyl chloride pipe with a 12.7- by 63.5-mm round-bottom, straight-wall, 1-bar high-flow porous ceramic cup (0652X11-B01M3, Soil moisture Equipment Corp., Santa Barbara, CA) affixed to the end. A stopper was sealed to the top of the cup with two lines of 3.175-mm o.d. nylon tubing passing through, one to the bottom of the cup, and the other protruding 1 cm into the top of the cup. The tubing to the upper end of the cup was used to evacuate the cup, and the tubing to the bottom of the cup was used to remove accumulated soil water. The cup was emptied, evacuated and emptied by alternately drawing a -30 kPa vacuum on the two nylon tubes. The polypropylene liner adhered to the rooting medium as it shrank and swelled, facilitating infiltration of water into the potting medium. Two calibrated Watermark sensors were installed at approximately 12.5 and 37.5 cm depths to monitor column soil water content and dictate irrigation scheduling.

The rooting medium used for the study was a mix of Mendon series clay loam soil (fine-silty, mixed, superactive, mesic Calcic Pachic Argixerolls) and Black Gold® Canadian sphagnum peat moss. The Ap horizon of Mendon series clay loam soil was collected at the USU Cyril Reed Funk Research Farm south of Richmond, Utah, USA (lat 41.89 N, long 111.81 W, altitude 1405 m a.s.l.). The Mendon soil was selected because of its low NO_3^- content (0.067 $mmol_c$ L^{-1}), low total N content (0.24%) and OM concentration of 5.5%. Peat moss was added to avoid compaction, improve porosity and facilitate infiltration. Rocks and larger plant residues were removed from the field-moist soil, and soil was mixed with peat moss in a concrete mixer in the proportion 2:1 soil to peat moss. The analysis of the soil-peat rooting medium is shown in Table 4-1. The initial N concentration of the mixture of soil and peat moss was 2.67 g kg^{-1}. The mixture of soil and peat moss was packed to a bulk density of approx. 1.30 Mg m^{-3} by hand with significant tamping and shaking using a small amount of water to obtain uniform packing. The four replications of each species were randomly assigned to one of 16 columns.

2.2 Column preparation and planting

Watermark sensors (Model 200SS, Irrometer Co. Inc, Riverside, CA, USA) were selected and used to determine when columns needed to be rewatered and calculate the amount of water to add. These sensors were selected because they respond rapidly and reliably to the range of variation in soil water status relevant to forage management, avoiding excessively frequent or intensive irrigation. Gravimetric calibration of

Watermark sensors was conducted at the USU Research Greenhouses before installation in soil columns. Two Watermark sensors were uniformly spaced in the center of a 1-gallon pot with the clay loam soil used in the columns. Initially, water was added to each pot until it reached field capacity, and resistance was recorded. After water was allowed to drain, pot weights were determined at the same time on successive days during the calibration. Volumetric water content measurements versus block resistances were plotted, and an equation was obtained for each pair of sensors, generating multiple calibration equations. These Watermark sensors were soaked overnight in water and installed wet into plant growth columns, as recommended by the manufacturer. Sensors were installed via an access hole made to the desired depth using a 12.7-mm-diameter PVC pipe. The access hole was filled with water and the sensor was seated at the bottom of the access hole. Then the access hole was filled with soil and tamped firmly, avoiding compacting the soil. Sensors from all columns were connected to a multiplexer (Model AM16/32) and datalogger (Model CR1000) to record resistance data (Campbell Scientific, Logan, Utah, USA). The datalogger was programmed to take hourly resistance readings and make temperature corrections of the sensors' readings.

Seeds of meadow bromegrass (*Bromus biebersteinii* Roem. & Schult., cv. Cache; MB, non-CT, non-N-fixing grass), inoculated cicer milkvetch (*Astragalus cicer* L., cv. Monarch; CMV, non-CT, N-fixing legume), inoculated birdsfoot trefoil (*Lotus corniculatus* L., cv. Langille; BFT, CT-containing, N-fixing legume), and small burnet (*Sanguisorba minor* Scop., cv. Delar; SB, HT-containing, non-N-fixing forb) were planted in each column around the suction lysimeter, and the top of the column was

covered with clear plastic wrap to encourage germination. Seedlings were thinned to 54 plants m^{-2} (3 plants per column), and irrigated during establishment to encourage deep root development. During the study, plants were treated with insecticides for aphids (*Talstar:* bifenthrin, 0.98 mL L^{-1}), thrips (*Enstar II:* S-kinoprene, 0.78 mL L^{-1}) and spider mites (*Floramite:* bifenazate, 0.65 mL L^{-1}; *Conserve*: spinosad, 0.78 mL L^{-1}; *Avid:* 0.31 mL L^{-1}) to combat infestations.

Growth conditions in the greenhouse unit during the study were 29/22°C day/night temperatures and 16-h daylength (6 AM to 10 PM). Average natural integrated daily photosynthetic photon flux density was 21 mol m^{-2} d^{-1}, ranged from 2 to 50 mol m^{-2} d^{-1}; and average supplemental lighting provided 9 mol m^{-2} d^{-1} between 6 a.m. and 10 p.m.

Feces and urine from cattle fed hay of the four treatment species were collected and frozen in autumn of 2016, and subsamples were freeze-dried and analyzed for NH_4^+, NO_3^-, dry matter, N, C and ^{15}N concentrations (Table 4-2; Table 4-3). Concentrations of the CT of BFT feces and the HT of SB feces were also determined; CMV and MB do not synthesize tannins.

2.3 Harvesting

Harvesting of herbage from columns began after 2 months of establishment. Plants were clipped to a 10-cm height above the soil surface on 19 January, 2 March, 19 April, 26 May, 6 July, 18 August, 30 September, and 31 October of 2017 and harvested herbage dry matter was frozen at -20°C and then freeze dried. Total herbage produced over the study period was determined by summing the amount produced at each harvest

on a per-column area basis and converting it to a per-ha basis. Thawed manure of a given

species was applied to columns of that forage species two times (20 January and 9 March

2017) after plants had regrown to maturity; maturity was defined as plants reaching a

"closed" canopy with 95% light interception and regrowth reached this stage in

approximately five weeks. Applications rates were 600 g of feces and 314 mL of urine,

equivalent to 1200-2600 kg N ha^{-1} for feces (Allen et al., 1996) and 300-1,500 kg N ha^{-1}

for urine (Haynes and Williams, 1993) depending on forage species. These rates

represent typical animal excreta deposition rates, on a mass or volume per area basis, and

were followed by irrigation water applications.

2.4 Irrigation

Irrigation applications were based on Watermark sensor readings. Resistance data

were recorded by the datalogger on a laptop computer between 23 September 2016 and

31 October 2017 and soil water potential was calculated during this period from each

sensor's resistance using the equation of Shock et al. (1998):

$$\text{For Model 200SS} \quad S = -\frac{(4.093 + 3.213\ R)}{1 - 0.009733\ R - 0.01205\ T}$$

Where S = soil water potential in kPa, R = resistance in k ohms; T = temperature in C.

Field capacity and permanent wilting point were defined for this clay-loam soil as

-12.5 kPa and -1,500 kPa, respectively (Werner, 1992), and a 50% of depletion of

available water between field capacity and wilting point was set as the criterion for irrigation. To determine the volume of water to add by irrigation, the calibration equation was used to convert resistance readings to column water volume for each column each day. This value was divided by the volume of that column's water holding capacity, and the volume of water needed to restore field capacity was added to all replications of a given plant species on the same day at each irrigation. Irrigation water was applied as a drip from IV bags hung from a rack above the columns. Each bag was filled with the volume of water needed and the valve of the bag was regulated to drip this water onto the column during the next 8 hours. Total irrigation water added was determined by summing the amount of water added to each column prior each harvest and converting per-column area to a per-ha basis.

Before each harvest, a leaching volume of 500 mL was added to the irrigation water volume, lysimeters located in the center of each column were used to remove the excess soil water, and the volume was recorded. These samples were frozen for N analysis. Concentrations of NO_3^- and NH_4^+ in these leaching fractions were determined using a flow injection colorimetric method (Lachat N Autoanalyzer: QuickChem 8500). Total N, NO_3^- and NH_4^+ leached from each column was determined by multiplying the concentration by the amount leached and converting column area to ha. Total leached N was the sum of leached NO_3^- and NH_4^+. Total of each leached component was determined by summing amounts for all harvests.

2.5 Destructive harvest

At the end of the study in November 2017, the four replicate columns of each species were destructively harvested as shoot development reached a closed canopy. Harvest was done during one day for each replication, and data were collected on root, crown, and herbage DM and N and C concentrations. Yield calculations were based on the soil surface area of the column. To distinguish crowns from repeatedly harvested herbage biomass, crowns were defined as shoots below 10 cm height, and were separated from roots below the lowest visible shoot. The entire crown was removed before the uppermost soil section was split vertically. Herbage and crowns were freeze-dried to constant weight and ground to pass the 1-mm screen of a Wiley mill (Thomas Scientific, Swedesboro, NJ).

2.5.1 Soil Sample Collection and Analysis

Soil columns were separated horizontally into 4 depths from the soil surface (0-10 cm, 10-30 cm, 30-60 cm, and 60-75 cm) using a saw. Each layer, including roots other than tap roots, was divided vertically into two halves. In one half of each layer, the total soil N, NO_3^-, NH_4^+ and C were determined. Soil subsamples were extracted immediately after sampling with 2M KCl (1:5 soil:solution w/w). Inorganic N pool size was calculated from NO_3^- and NH_4^+ in soil KCl extracts using a flow injection colorimetric method (Lachat N Autoanalyzer: QuickChem 8500). Total N, NO_3^-, NH_4^+ and C on a per-ha basis were calculated by multiplying their concentrations and the amount of soil at the respective soil depth, considering the initial bulk density (1.3 Mg m^{-3}), and then summing

the values for all four soil depths. Moist soil samples were sieved to pass a 2-mm screen

and stored at 4°C or air-dried for other measurements. Soil moisture content of each

sample was measured by oven drying of a 10-g subsample at 105°C for 48 h. For soil N

and C analysis, air-dried soil was finely ground and sieved (0.25 mm sieve). Total soil N

was determined by the dry combustion method using a Skalar Primacs[SN] Nitrogen

Analyzer and total soil C was determined by dry combustion using a Skalar Primacs[SLC]

Carbon Analyzer. Because the final soil pH was 7.2, an acid test was used to confirm free

carbonates by placing a drop of dilute acid (10% HCl) onto 1 g soil. The absence of

bubbles (effervesce) from released carbon dioxide demonstrated that carbonates were not

measurable, so total soil carbon was used as an indicator of soil organic carbon

concentration.

2.5.2 Root Sample Collection and Analysis

Roots from the other vertical half of each soil layer were separated from the soil,

washed and collected using a root washer (GVF Hydropneumatic Elutriation System,

Gillison's Variety Fabrication, Inc., Benzonia, MI, USA) (Smucker et al., 1982).

Taproots were not split and were included in this half. Three sieves progressing from

coarse to fine allowed the collection of large, medium and fine roots. Cleaned roots were

stored at 4°C in 10% v/v aqueous isopropyl-alcohol until they were scanned and analyzed

using WinRHIZO™ software. Roots were placed in a glass tray of water, spread without

overlap and roots were identified as colored lines coded according to root diameter. In

medium and fine roots, despite thorough washing, peat moss could not be readily

separated from roots, requiring addition steps. Roots with attached peat were stirred in a cup for 10 minutes in order to homogeneize the sample and cause the roots and peat moss to mix thoroughly. The subsample was quickly decanted to a glass tray and subsampled. These roots plus peat were spread in the tray, scanned and the image analyzed. Before scanning, filters were established using the software that allowed us to ignore non-root material like bubbles and peat moss. One stirred subsample was evaluated per species, per layer, per replication and root size. The results from this stirred subsample were used to identify the peat in all the other medium and fine root samples.This subsample was weighed, dried in the oven for 24 hours at 80°C, and DM recorded. The remaining roots plus peat were also weighted and oven-dried. The specific root length density (RLD) and root surface density (RSD) with the dry weight of the subsample was extrapolated to the total dry weight of the whole sample in order to get the total RLD and RSD. Large roots were dried in a forced air oven at 70°C for 48 h, weighed, and finely pulverized in a ball mill (Retsch, Haan, Germany) using a frequency of 30 Hz for 5 minutes before determining total root N and C concentration by the dry combustion method using a Skalar Primacs^{SN} Nitrogen Analyzer and Skalar Primacs^{SLC} Carbon Analyzer, respectively. Total root length, root surface area and root dry matter on a per-ha basis were calculated by multiplying their concentrations by the volume of soil at the respective soil depths, and then summing the four soil layers.

2.5.3 Herbage and Crown Sample Collection and Analysis

Herbage and crown samples were frozen using dry ice during destructive harvest in the greenhouse, then stored at -20°C until freeze-dried. Samples were ground to pass the 1-mm screen of a Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA) and stored in sealed plastic bags until total N determinations using a Skalar PrimacsSN Nitrogen Analyzer. Herbage DM collected during all eight harvests was sampled for N concentration after DM production was determined.

The ^{15}N natural abundance method was used to determine herbage N_2 fixation because it is considered to be more reliable and precise than other methods (Danso, 1995). It was assumed that the N isotope composition of roots and crowns was similar to that of herbage N (Heichel et al.,1984). The same principles and equations that were used in the field pasture study (Chapter 2) were applied here. Herbage samples of the four forage species were collected at the beginning of the study (19 January), from the middle harvest (26 May) and at the end of the study (31 October) for ^{15}N concentration determined by mass spectrometry. The ^{15}N natural abundance was calculated using the methods of Shearer and Kohl (1986),

$\delta^{15}N$ = $\underline{\text{R sample – R standard}}$ * 1000

                 R standard

where:

R = $^{15}N / (^{14}N + ^{15}N)$

R standard = R air = 0.3663 atoms % ^{15}N

$\delta^{15}N$ = Parts per thousand deviation from the $^{15}N/^{14}N$ ratio of atmospheric N_2.

According to Amarger et al. (1979) and Kohl et al. (1980), the proportion of fixed N

$(P_{fix}) = 100 \ (x - y) \ / \ (x - c)$

where:

P_{fix} = the proportion of BFT and CMV N derived from N_2 fixation,

x = the mean $\delta^{15}N$ of the total N of the non-N_2 fixing reference plant (SB) where N

requirements were obtained from the pool of soil mineral N,

y = the mean $\delta^{15}N$ of the shoot N of BFT and CMV samples,

c = the isotopic fractionation which occurs during N_2 fixation, derived from the $\delta^{15}N$ of

the total N of BFT and CMV plants grown from seed in sand culture and therefore

obtaining all their N from symbiotic N_2 fixation and used to calculate ^{15}N discrimination

in the N_2-fixing plant (Evans, 2001). For BFT, this value was -4.32‰, and -1.34‰ for

CMV.

      The amount of N_2 fixed for each plant component or each legume species was the

product of P_{fix} and the amount of N in the plant (kg ha^{-1}). Extreme care was taken to

ensure that root samples were not lost during the washing and scanning processes, and

that cross-contamination among samples did not occur during the weighing step for ^{15}N

enrichment determinations. A quadratic equation of P_{fix} for BFT and for CMV was

developed using values for the three measured dates (19 January, 26 May and 31

October) and used to calculate herbage BNF for all eight harvests.

The CT concentration of BFT herbage from all eight harvests was determined using the method of Grabber et al. (2013) and the HT concentration of SB was determined using the method of Hartzfeld et al. (2002).

2.6 Nitrogen balances

A N mass balance on a per-ha basis was constructed using N sources and sinks for simulated pastures of BFT, CMV, MB and SB grazed by beef cattle (Keeney, 1979). Nitrogen sources comprised N_2 fixation measured for legumes, N added in manure applications, and soil organic matter N and soil inorganic N when columns were packed. Nitrogen sinks comprised soil organic and inorganic N and N accumulated in herbage, crowns, and roots by the end of the study (Scholefield et al., 1991). Gaseous losses by denitrification were considered to be negligible because chemical N fertilizer was not applied, and soil moisture was maintained at or below field capacity during the study. Volatilization from decomposing herbage was also considered to be negligible since all herbage above 10 cm was collected at each harvest date. However, the proportion of urine N volatilized as gaseous ammonia was estimated as 15% (Ryden et al., 1987; Vertregt and Rutgers, 1987; Lockyer and Whitehead, 1990) based on the soil and temperature regimen maintained in the greenhouse. The proportion of feces N volatilized as ammonia was assumed to be 3% (Ryden et al., 1987). Nitrogen in herbage harvested at 5-week intervals and N in the leaching fraction before each harvest were included as N sinks. To estimate N gained or lost from a given forage species column system, the summed initial N sources were subtracted from the summed final N sinks.

2.7 Statistical analysis

Variables from the column study were analyzed as a randomized complete block

design with four replicates as blocks; species was a fixed factor and replicates was a

random factor. For responses repeatedly measured over harvest times or over soil depths,

the model also contained harvest or depth and harvest*species or depth*species

interactions as fixed factors and replication*species as a random factor. A heterogeneous

first-order autoregressive error structure was used for repeated measures on each

experimental unit. Differences among the treatment least squares means (LSMeans) were

tested using pairwise comparisons with Tukey-Kramer's method to adjust multiplicity.

LSMeans and standard errors of responses that were log-transformed for analysis were

back-transformed (exponentiated) for reporting. LSMeans of response totals differed

from the numeric sum of the LSMeans for layers of the same species because the model

for LSMeans of totals was adjusted for variation among total values for each replication.

The greater the variation among replications, the greater the difference between the

LSMean for a species' total relative to the numeric sum of layer LSMeans. All analyses

were conducted using PROC GLIMMIX in SAS/STAT 15.1 (SAS Institute, Cary, NC,

USA). Statistical significance was specified as $\alpha = 0.05$.

3 RESULTS

3.1 Herbage dry matter and N concentration

In this year-long study, plants were maintained at a growing season temperature

and light regimen, and all four species were harvested on the same dates but irrigated

between harvests according to their water use. Birdsfoot trefoil produced more herbage

DM than CMV and MB but BFT did not differ from SB, which did not differ from CMV

and MB (Table 4-4).

Harvest effects: Herbage DM and N concentrations were strongly influenced by

species and harvest but both variables showed a species by harvest interaction

(P=0.0004). Meadow bromegrass DM production did not differ among harvests (Table 4-

4). Both legume forages produced greater DM at the first harvest and least at the last

destructive harvest (BFT: P<0.10; CMV<0.0001). Similarly, SB showed greatest

production of DM at the first harvest, but DM production was least following application

of manure, in March and April (P<0.0001). The herbage N concentration of all four

species was least at the first harvest (Table 4-5). By the end of the study, BFT had

accumulated nearly twice as much herbage N as the other three species (Table 4-6).

3.2 Watermark sensor measurements

Watermark sensors continuously measured soil electrical resistance over the

course of the study and were used to calculate soil water potential for two depths in each

column. Wetting and drying trends of the soil for each species averaged for the four reps

are shown in Figure 4-1. Saturation of the lower root zone was prevented by drip

irrigating with no more water than the soil could hold at field capacity. Watermark

sensors responded to irrigation within one hour. After the first harvest, BFT dried the

upper soil (0-25 cm) more than the lower soil (25-50 cm). In contrast, CMV water uptake

was greater from the lower soil after the first harvest, after which it drew water similarly

from the two layers until the last months of the study when water uptake of CMV was greater from the upper than the lower soil. Meadow bromegrass water uptake was greater from the upper soil throughout the study, while SB water uptake shifted from the upper soil at the beginning of the study to the lower soil for the balance of the study. The lower soil of MB and SB columns was saturated at the last harvest. Average sensor readings for each species at both depths can be found in Figure 4-2. In general, BFT, CMV and MB appeared to draw water preferentially from the upper than from the lower soil profile, while SB used water from both soil depths about equally.

3.3 Plant water use

Water use was was similar for BFT, SB and MB, and greater than for CMV, (Table 4-7). Water use between harvests was strongly influenced by species and by harvest, and a species by harvest interaction was observed (P=0.0008). Columns were not irrigated until the first harvest at 118 days to encourage plants to root deeply, which accounts for the greater volume of water that was applied at this harvest.

3.4 Leached N

A 500-mL leaching fraction was added to each column before each harvest so leachate could be extracted using suction lysimeters to determine mineralization of NH_4^+ and NO_3^- from leachate volume and concentration (Table 4-8). Forage species influenced total NH_4^+ and NO_3^- leached; SB leached the most NH_4^+ while CMV leached the least. Birdsfoot trefoil leached the most NO_3^- while MB leached the least.

3.5 Soil nitrogen and carbon

Soil organic N and C concentrations (Table 4-9) and per-ha totals (Table 4-10) were measured at the end of the study. Soil N and C concentrations of all species were significantly greater in the shallowest layer than in the deeper soil layers ($P<0.0001$). In the deepest soil layer (60-75 cm), SB, which commonly withdrew more water from the lower soil than other species, had greater soil organic C concentrations than BFT. Greater amounts of organic N ($P<0.0001$) and organic C ($P<0.0001$) were detected in 30-60 cm soil layer (Table 4-10). Total soil organic N was greater ($P<0.0001$) for MB and SB than for BFT and CMV, which fixed their own N. Total soil organic C was greater ($P=0.0681$) for BFT than MB, but BFT did not differ from CMV and SB. Soil NH_4^+ concentration was greater in the deepest than in the shallowest soil layer while soil NO_3^- concentration was greatest in the shallowest layer and decreased with depth for all species (Table 4-11). There were no differences among species for total soil NH_4^+ and NO_3^- (Table 4-12).

3.6 Root length density, root surface density and root biomass

Neither total root length ($P=0.2276$) nor total root surface ($P=0.2586$) differed by species (Table 4-14), but their concentrations, RLD and RSD, differed significantly for species at some depths ($P<0.001$). A species by depth interaction was observed and both metrics differed among species in the same way at each depth (Table 4-13; Fig. 4-3). At 0-10 cm, root length was greater for SB and MB than CMV and did not differ from BFT (Table 4-14). At 10-30 cm, root length was greater for SB than BFT and did not differ from MB or CMV. Species did not differ at 30-60 cm and 60-75 cm. The root surface

area of species did not differ in the 0-10 cm or 30-60 cm layers, but from 10-30 cm it was

greater for SB than BFT but SB did not differ from CMV and MB, and at 60-75 cm, MB

was greater than SB, and did not differ from BFT and CMV.

Soil concentration of root DM was influenced by both species and depth, with a

strong interaction between them ($P<0.0001$) (Table 4-15). In the shallowest soil layer,

root DM concentration was greater for MB and SB than BFT and CMV ($P<0.0001$). The

DM of the crown of plants, however, where there was considerable DM, was determined

separately from root and shoot DM. Root dry matter within layers was greater in the

upper 30 cm of the soil than in the deeper 45 cm of the soil ($P<0.0001$). The total root

DM of MB was greater for BFT and CMV but was not different from SB (Table 4-16).

3.7 Root N and C composition

Root N concentrations differed among species at lower but not upper depths

where it was greater for BFT than other species (Table 4-17), creating a species by depth

interaction ($P=0.0059$). Within species, the root N concentration of BFT was greater in

roots in the deepest layer than in the uppermost layer and intermediate between 10 and 60

cm, while for CMV and MB, root N was greater in the uppermost soil layer than the

deepest soil, and did not differ with depth for SB. Root C concentration was not affected

by depth for a given species,but differed among species ($P=0.01$), with all forbs having a

greater C concentration than the grass (Table 4-17). When root N on a per-ha basis was

totaled, MB columns contained more root N than BFT and CMV columns but did not

differ from SB (Table 4-18). Similarly, MB columns accumulated more root C than BFT and CMV columns and did not differ from SB (Table 4-18).

3.8 N$_2$ fixation in legume species

The δ^{15}N natural abundance was assessed at the beginning, middle and end of the study, and was significantly affected by species and harvest (Table 4-19). At the beginning of the study, the δ^{15}N of BFT was less than for MB and SB and did not differ from CMV, confirming that N$_2$ fixation was occurring in the legumes. Variation at the other two harvests was too high for differences to be observed among species. At the first harvest, over half of BFT N was from fixation (P$_{fix}$) while only 2% of CMV N was from fixation. In the middle of the study period, following fertilization with manure, CMV had stopped fixing N$_2$, while BFT had reduced BNF and only derived 10.5% of N from fixation. By the end of the study, both legumes were deriving most of their N from N$_2$ fixation. Over the course of the study, BFT herbage accumulated more total symbiotically fixed N than CMV (Table 4-20).

3.9 Tannin concentration in forage species

The tannin concentration was measured in shoots of all four species, including CMV and MB which are not reported to contain tannins (Figure 4-4). Mean CT concentrations in BFT ranged from 9 to 16 g kg^{-1} DM, while in SB, HT concentrations ranged from 18 to 26 g kg^{-1} DM.

3.10 Partitioning of DM, total N and fixed N among plant components at destructive harvest

To create Table 4-21, values for DM and N concentrations of herbage regrowth since the previous harvest, and for crowns and roots accumulated during the study, were determined at destructive harvest. Values were compared statistically (within and among species) and total N was calculated by multiplying DM by N concentration for herbage, crowns and roots. The total plant DM of MB and SB did not differ at the destructive harvest and were greater than for BFT and CMV (Table 4-21). Proportion of total DM and total N within each species at destructive harvest was calculated by dividing the total DM or N of each component by the total value for each species. BFT allocated a greater proportion of DM to herbage than MB; and MB allocated a greater proportion of resources to root DM than BFT (Fig. 4-5). Approximately half of plant DM was invested in crowns of all species.

Nitrogen concentrations of roots and crowns were less than for herbage of all species (Table 4-21). The N concentrations herbage and roots did not differ among species, but the crown N concentration was greater for CMV than that of BFT but neither differed from MB and SB. Meadow bromegrass allocated a greater proportion of N to the roots than BFT, while CMV and SB were intermediate (Fig. 4-6). By contrast, BFT allocated a greater proportion of N to herbage than MB and SB, while CMV was intermediate. All four species allocated the same proportion of N to crowns.

The accumulation of whole-plant N was greater in MB and SB than in CMV and did not differ from BFT (Table 4-21). Herbage N accumulation was greater for BFT, SB

and MB than for CMV, while crown N accumulation was greater for MB and SB than for BFT and CMV. Root N accumulation was greater for MB and SB than for CMV and but did not differ from BFT.

Accumulation of fixed N among plant components of BFT and CMV at destructive harvest is shown in Table 4-22. The proportion of N derived from N_2 fixation allocated to crowns and roots was based on herbage fixation rates estimated for all eight 2017 harvests dates using quadratic equations for P_{fix} for each species derived from the three calculated P_{fix} values reported in Table 4-19. Birdsfoot trefoil fixed three times more N than CMV. Total fixed N was allocated to crown and roots based on their N concentrations. Both legumes averaged 19, 20 and 56% of fixed N in roots, crowns and herbage, respectively.

3.11 N balances

Table 4-23 gives the N balances for the four simulated grazing systems during the study. Initial organic and inorganic N of the soil-peat planting medium were considered N sources. Other N inputs were from applications of manure and symbiotically fixed N_2 in BFT and CMV. The organic and inorganic N of the planting medium were viewed as N sinks along with leached and volatilized N and the total N in herbage, crowns and roots. Herbage production during the study was greater for BFT than CMV, and N_2 fixation provided 3.5 times more N from fixation for BFT than CMV. The N from manure was about equally from urine and feces except for SB, where more N came from feces than urine; this is believed to be an effect of the HT in SB.

Final soil inorganic N did not statistically differ among species for soil NH_4^+ or NO_3^-, so the mean for the four species was reported in Table 4-23. Total herbage biomass N harvested during the study was greater for BFT than for other species. Nitrogen immobilized in crowns over the course of the study was greater for SB and MB than for the two legumes, and MB accumulated more root N than BFT and CMV. Numerically less N volatilization was estimated for SB because 80% of volatilized N came from the urine of cattle fed BFT, CMV and MB hays, while just 40% came from the urine of SB hay-fed cows. The total leached N contributed the least to N outputs of the four simulated grazing systems; it did not statistically differ among species, so the mean for the four species was reported, averaging 1 kg N ha^{-1}.

Nitrogen balances were calculated by summing initial N sources and subtracting final N in sinks. Negative values represented a decrease in system N and positive values represented N added to the system by forages. Surprisingly, total N decreased in legume systems while non-legume systems gained N.

4 DISCUSSION

The present study was conducted to investigate N cycling and the contributions of representative deep-rooted perennial legumes, a cool-season grass and a forb that are well-adapted to production in temperate climates under irrigation on alkaline soils, to soil N and C in pasture systems. Contributions of N came from the N_2 fixation and ruminant waste, and N was accumulated in herbage, crowns and plant roots, as organic and inorganic soil N, and small amounts were leached or volatilized during a year of growth

and regrowth cycles the approximated forage establishment and two to three years of production. The study was conducted in a greenhouse with a well-controlled environment in 1-m-deep columns to exclude the variability of N deposition in pastures.

4.1 Water use

In a similar column study, Reynolds (2010) found that the legumes BFT and white clover and the cool-season grasses orchardgrass and tall fescue withdrew more water from an upper (10-20 cm) layer of the soil profile than from lower soil layers (40-50 and 70-80 cm), while SB water use did not differ statistically among layers. In the current study, BFT and MB also absorbed more water from the upper than the lower soil and SB withdrew soil water more equally throughout the soil profile. Grieu et al. (2001) found that the ability of a plant to extract water is more related to plant growth than the development of the root system. In the current study, CMV produced less DM and used less irrigation water than all other species. Jensen et al. (2001) found a linear response between DM production of MB and water use, and that was more generally the case in the present study, with BFT using more water than other species but also producing more DM.

Water-use efficiency (WUE) can be expressed as shoot DM L^{-1} water consumed and herbage DM produced. It is challenging to accurately measure the WUE of deep-rooted legumes in the field because they can usually root more deeply than soil water use can be measured. However, in this study, all herbage DM was collected and all applied water was measured (Tables 4-4 and 4-7). The WUE of the herbage DM production of

BFT and CMV was 1.23 and 1.30 kg L^{-1}, respectively, while the WUE of MB and SB was 0.86 and 0.90 kg L^{-1}, respectively. Therefore, the WUE of the legumes was approximately 40-50% greater than the WUE of the grass and the forb, because less photosynthate was invested by legumes in belowground DM and therefore more could be invested in shoot growth.

4.2 Root growth

The growth and development of plant shoots depend on adequate root growth for uptake and assimilation of nutrients and water, but root DM accumulation is an energy cost to the plant. In this study, root DM was four fold greater for MB than for BFT and five fold greater for MB than for CMV, and SB root DM was approximately two times greater than BFT and three time greater than CMV (Table 4-16). Differences in root DM did not reflect water uptake strategy within the soil profile; however, Watermark sensor data for BFT and MB show that they both extracted more water from the upper than the lower soil layers, while sensor data showed that CMV and SB maintained similar water potentials in upper and lower soil layers. The uppermost layer of soil is where the majority of soil nutrients and microbes reside, and where root biomass and its exudates are most readily decomposed by oxygen-dependent soil microbes (Lynch and Bragg, 1985). The grass MB invested nearly 80% of root DM in the uppermost 10 cm of the soil profile and the other 23% was distributed in decreasing amounts with greater soil depth. Among the four species, CMV invested 7% and MB 4% of total root DM in the bottom 15 cm of the soil column.

While RLD and RSD, which are the root metrics most relevant for soil water uptake, were generally greater in the uppermost soil layer at the end of the study (Table 4-13), there were no differences among species for total root length and total root surface area (Table 4-14) while total root DM differed significantly among species (Table 4-16).

Small burnet invested more than half of its root DM in the 20 cm between 10 and 30 cm deep in the soil profile, and a little more than one-third of root DM in the upper 10 cm, which means that 90% of root DM was located in the upper half of the soil profile Table 4-16). However, SB RLD and RSD were greater in the upper 10 cm than the bottom 15 cm, while the RLD and RSD of SB in the 10-30 and the 30-60 cm layers of the soil column were intermediate and did not differ. This illustrates that root storage (DM) and root water uptake occur in different layers. Early in the study, water potential of the upper SB Watermark sensor was less than that of the lower sensor, but for the balance of the study, water potential was similar for both sensors, suggesting effective use of water throughout the soil column. This is supported by RLD and RSD data.

Birdsfoot trefoil also invested about half of its root DM in the 10-30 cm soil layer and another third in the upper 10 cm. During establishment, which is relatively slow in BFT, water was used about equally from the upper and lower soil layers. Following establishment, the upper sensor registered significantly lower water potentials for the balance of the study than the lower sensor. The close coordination between change in water potential of the two sensors suggests that the plant was being supplied with deep water at night. The RLD and RSD were greater between 30 and 75 cm depth than at 10-30 cm, also suggesting that the lower soil was well-supplied with roots for water uptake.

Cicer milkvetch invested half of its root DM in the upper 10 cm of the soil and the other 50% of root DM was present in diminishing amounts with depth. Cicer milkvetch is a rhizomatous plant species, spreading by underground stems that concentrate near the soil surface to facilitate lateral spread of the plant shoot. Cicer milkvetch used about half as much water as BFT, and the upper portion of the soil did not become more depleted than the lower soil until the last quarter of the study. However, the RLD and RSD of CMV did not vary among soil layers. Overall, there were no significant correlations of the water use of these plant species and their total root DM, total root length or total root surface area.

More than three-quarters of the root DM of MB was concentrated in the uppermost 10 cm of the soil and, like CMV, root DM diminished with depth. The RLD and RSD, however, decreased by about 35% in the layer between 10 and 30 cm in depth compared with the upper 10 cm, then increased again with increasing depth, which was also similar to change in RLD and RSD for all other species. This is not the pattern typically found in pasture soils, where RLD or RSD decrease with rooting depth (e.g., Greenwood and Hutchinson, 1998), and may therefore be an artifact of limiting rooting depth to 1 m.

Water potential profiles of the two BFT and SB sensors, which are both tap-rooted species, were similar as were profiles of the two MB and CMV sensors. Meadow brome has a fibrous root system and CMV spreads by rhizomes and therefore is not strongly tap-rooted.

4.3 NO$_3^-$ leaching potential

Earlier research studies (Minns et al., 2001; Scherer-Lorenzen et al., 2003)
reported that leaching was negatively correlated with root biomass, and elevated RLD
was associated with greater water and nutrient uptake (Grieu et al., 2001). In this study, N
was deliberately leached prior to each harvest to determine differences among species in
net N mineralization, and a negative correlation of leached N with RLD was not found.
From greatest to least, leached NO$_3^-$ by each forage species was BFT > CMV > SB > MB
while leached NH$_4^+$ by species was SB > MB > BFT > CMV; the average leached NH$_4^+$
for all species exceeded the average leached NO$_3^-$ (Table 4-8).

The same volumes of urine and feces were applied to each soil column to
replicate known volumns of waste deposition during grazing. These applications were
only made twice: a week after the first and second harvests. The order of urine N
concentration was CMV > MB > BFT > SB and the order of feces N concentration was
SB > CMV > BFT > MB. The SB feces contained measurable HT which is thought to
suppress mineralization, but NH$_4^+$ leaching does not reflect this expectation (Table 4-8).
Santos et al. (2013) found a direct relationship between root and shoot growth and
inorganic N uptake in a grass, and the NO$_3^-$ concentration was indeed lower in MB
leachate than for the legumes and forb, but NH$_4^+$ concentration of MB leachate was
greater than that of the legumes, suggesting more active mineralization of soil OM for
MB than for the legumes. Legumes were able to use BNF and therefore may have
interacted less with rhizosphere microbes in support of SOM mineralization than was the
case for MB or SB.

Some NO_3^- leaching is thought to be inevitable soils supporting legumes (Tilman et al., 1996), even when N is supplied by biological N_2 fixation (Macduff et al., 1990); legume roots and leaf litter are rich sources of protein, so decomposition and mineralization of legume leaves releases more N than with other species (Ledgard and Giller, 1995). Far more N was applied as manure than was supplied by BNF in the present study (Ryden et al., 1984). The N in urine is readily available as urea (Haynes and Williams, 1993; Whitehead, 1995) so this N will be used before feces N, which must be mineralized before uptake (Menneer et al., 2004). It should be noted that N leaching is reported in g/ha (Table 4-8) which is a neglibile amount.

4.4 Root N and C concentrations

In this study, herbage dry matter above 10 cm was harvested every five weeks which allowed sufficient time for plants to completely recover root N and C reserves between harvests (Vance et al., 1979; Kim et al., 1993).

While the C concentration of roots did not differ by depth for a given forage species, the root C concentration of MB was less at every depth than that of other species (Table 4-17). This was likely due to differences among species in the starches and sugars accumulated in their root systems as seen by Volenec et al., (1991) in different lines of alfalfa. Small burnet is recognized as a species with high potential carbohydrate storage because of its prominent taproot (Ogle, 2002) as is CMV, which consists of hardy underground crowns and prolific rhizomes (Acharya et al., 2006). Birdsfoot trefoil does not replenish the storage carbohydrates used for spring growth until late summer or early

autumn (Smith, 1962) but the C concentration in BFT roots in this study was equal to that

of CMV and SB, perhaps because of the length of the interval between harvests. Grasses

such as MB have deep fibrous root systems that contribute to soil organic C through root

turnover following shoot harvest (Conant, 2010), but fibrous root systems have little

carbohydrate storage capacity compared with taproot systems.

4.5 Soil N and C concentrations and accumulations

Although a large addition of OM is required to significantly increase the pool of

soil OM, forage root turnover in the shallowest soil layer (0-10 cm) over the course of

this study resulted in greater organic N and C concentrations in this layer compared with

the rest of the soil column for all forage species (Table 4-9). In contrast to Ta et al.

(1986), a study carried out for five harvests in 15-cm pots, RLD and RSD of legumes in

the current study was not as great as for the grass and the non-legume forb, and so did not

contribute more N to this layer via N_2 fixation than were contributed by MB and SB.

Compared with accumulated soil organic N, mineralized soil inorganic N was

inconsequential in the present study, resulting in minimal leaching and denitrification

losses (Walley et al., 1996).

The greater soil organic C and N concentrations in the 0-10 cm soil layer at the

end of the study included OM added as feces and urine after the first and second harvests

(Haynes and Williams, 1993; Habteselassie et al., 2006a; Habteselassie et al., 2006b;

Russelle, 2008), which would support greater soil microbial biomass and mineralization

and thereby increase root proliferation (Dietzel et al., 2017). Root and manure additions

did not appear to cause soil organic N, which averaged 2.8 g kg^{-1}, to diverge, while soil organic C concentrations only differed the layer from 60-75 cm, where SB accumulated more organic C than BFT (Table 4-9).

Organic N sources such as roots and manure slowly release NH$_4^+$ (Shi et al., 2004) while improving microbial diversity and soil enzyme activities (Ouyang, 2016), reducing undesirable environmental impacts associated with urine and chemical N fertilizer. Mineralization regulates availability of organic N in soils (Schimel and Bennett, 2004), while nitrification, the biological oxidation of NH$_4^+$ to NO$_2^-$ or NO$_3^-$ (Prosser, 1989), is regulated by the availability of substrate NH$_4^+$ (Niklaus et al., 2001; Robertson and Groffman, 2015) which depends on the C:N ratio in SOM which in turn affects microbial activity (Booth et al., 2005). Soil C concentration has a role in controlling N cycling in soils by suppressing microbial activity as the C:N ratio increases (Rothrock and Hargrove, 1988). In the present study, manure from cows fed BFT, CMV and SB had C:N ratios less than 20, which supports net N mineralization, while manure from cows fed MB had a C:N of 27, possibly leading to immobilization of N (Robertson and Groffman, 2015).

There were no significant differences among species in soil inorganic N (Table 4-12). Tannins reduce urinary N and increase fecal N in waste (Woodward et al., 2009; Stewart et al., 2019) and HT slows nutrient mineralization of feces and SOM, nutrient recycling and nitrification (Baldwin et al., 1983; Hättenschwiler and Vitousek, 2000; Kraus et al., 2003). In this study, MB or SB had greater RLD and RSD in some soil layers while BFT, CMV or SB had the least, depending on depth; MB exceeded other

species for both in the deepest soil layer. In studies of cottonwood, the tannin

concentration in leaf litter was correlated with fine root production, since more root

length is needed to extract soil N that is less available due to increased soil tannin

(Fischer et al., 2006). For SB, the organic N applied as feces was greater and the N

applied as urine was less than for other species, but the tannin added with feces was also

greater for SB than for other species (Table 4-3). The greater tannin concentration may

have inhibited feces mineralization sufficiently to have increased effective rooting of SB.

Similarly, MB did not have an internal source of N and depended on root growth to

scavange available N.

Averaged across species, a smaller concentration of soil NH_4^+ than NO_3^- was

found in the shallowest soil layer (0-10 cm) (Shi et al., 2004) and the greatest NH_4^+

concentrations were found in the deepest soil layer (60-75 cm) (Table 4-11) suggesting

that NH_4^+ in the warm, oxygenated upper layer was used as it was generated by

mineralization (Booth et al., 2005). Similar preferential NH_4^+ uptake has been reported in

other studies (Marschner, 2012). Increased soil NO_3^- has been attributed to greater

nitrifier activity (Niklaus et al., 2001) resulting from manure additions (Müller et al.,

2003; Habteselassie et al., 2006a). Microbes generally prefer NH_4^+ to NO_3^- for their

growth, leading to transient immobilization of NH_4^+ (Recous et al., 1990), negatively

impacting nitrification rates.

Soil N status regulates the growth of pasture grasses (Stratton and Rechcigl, 1998)

and elevated soil N is thought to suppress legume BNF (Alston and Graham, 1982;

Ledgard and Steele, 1992). Indeed, the BNF of both BFT and CMV were suppressed

following the two manure applications but recovered by the end of the study (Table 4-19). Accumulation of total soil organic N at the end of this study was greater for the two non-legumes (MB and SB), while accumulation of total soil organic C was greatest for BFT and least for MB (Table 4-10).

4.6 Forage growth and N concentrations

Birdsfoot trefoil produced more herbage DM than CMV and MB during the study period (Table 4-4), and BFT water use was no different than for MB and SB (Table 4-7), but total BFT DM accumulation (shoots, crown and roots) at destructive harvest was less than for MB and SB (Table 4-21). However, at the final harvest, 33 kg BFT herbage DM was produced for every kg of N_2 fixed by BFT, while 45 kg of CMV herbage DM was produced for every kg of N_2 fixed by CMV (Tables 4-21 and 4-19). The proportion of BFT BFN to herbage DM production was similar for the early and mid-study dates. Clearly, BNF was not a drag on BFT herbage DM production.

In contrast, CMV produced the least total DM over the course of the study, and consumed less water than other forages. However, CMV resulted in more residual soil inorganic NO_3^- than the grass (Table 4-12), likely explained by reduced N plant uptake due to a relatively slow rate of plant DM accumulation.

The total DM yield of MB and SB at destructive harvest, which depend on N in the soil solution for their growth, was greater than that of BFT and CMV (Table 4-21). In this column study, manure was the main source of N for these species, and supported greater crown and root development. The two legumes were also fertilized with manure

which suppressed BNF in both but which seems to have been used by BFT for herbage production while CMV feces seem to have been mineralized but not used, since CMV soil NO_3^- and NH_4^+ totals were both elevated compared with SB for NH_4^+ or with MB, BFT and SB for NO_3^-.

4.7 N_2 fixation in legume forages

Legumes accumulate a greater ratio of ^{14}N to ^{15}N in shoot DM than grasses, indicating N_2 fixation (Vitousek et al., 1989). Biological N_2 fixation is used to support legume growth and metabolism, including photosynthesis, which generates the carbohydrates used in respiration that supplies energy for fixation (Marschner, 2012). It was documented in other studies that BNF of BFT is more sensitive than CMV to elevated soil inorganic N from manure application (Russelle and Buzicky, 1988). Similarly, Mallarino and Wedin (1990) found that BNF was more sensitive to high soil N availability in BFT than in clovers. In the present study, BFT fixed more N_2 than the non-tannin containing legume CMV at all examined harvests (Table 4-19). It's possible that this apparent discrepancy is due to greater genetic yield potential of BFT compared with CMV and greater utilization of soil inorganic N by BFT than by CMV, so that the period of inhibition of BFT BNF is short-lived and mitigated by BFT root uptake of both NO_3^- and NH_4^+.

4.8 Partitioning of N derived from N$_2$ fixation in legume species

In previous studies (Heichel et al., 1984; Heichel et al., 1985; Kim et al., 1993) it was demonstrated that most of the N$_2$ fixed by legumes was distributed to herbage (leaves plus stems). Similarly, Ta et al. (1986) indicated rapid transport of assimilated N to legume shoots, while Bergersen and Turner (1983) estimated that the total N in roots of subterranean clover (*Trifolium subterraneum*) represented a significant proportion of fixed legume N. In this study, greater proportions of fixed N$_2$ were found in herbage of BFT and CMV. Our results can also be compared with those of Russelle et al. (1994), who estimated that approximately 54% of fixed N was located in the herbage and crown of effectively nodulated alfalfa plants while 47% was located in the root system. Likewise, N proportions for BFT and CMV were similar to those of alfalfa reported by Volenec et al. (1991), who concluded that up to 50% of soluble root and crown N was utilized in shoot regrowth. Walley et al. (1996) also reported that 56% of fixed N in alfalfa was allocated to aboveground plant components.

4.9 Seasonal variation of tannins in BFT and SB

In this study, tannins did not fluctuate significantly among harvests, but appeared to be elevated when plants were younger, similar to results of Theodoridou et al. (2011) for sainfoin. Herbage was removed at every harvest, so it was not possible to evaluate the impacts on soil mineralization of CT and HT from litter decomposition. Tannins concentrations did not differ among harvests but for BFT, CT concentrations trended

higher as yields trended lower and vice versa, suggesting dilution of CT with greater DM yield, while HT concentrations of SB trended higher along with yield.

4.10 Nitrogen balances

A comprehensive accounting of organic and inorganic N sources and sinks allowed N gain or loss in BFT, CMV, MB and SB pasture systems to be determined by subtracting total sinks from total sources (Table 4-23). Nitrogen inputs to each system were similar and differed mainly by the addition of fixed N_2 in legumes. Nitrogen accumulated as soil organic N in MB and SB systems while the N accumulated in plant herbage, crown and roots was less than the N contributed by feces and urine. Nitrogen fixation decreases as the availability of soil N increases (Menneer et al., 2003), and reduced BNF following manure application as well as negative accumulation values for the two legumes suggest that this regulation was functioning well in these pasture systems. Vigorous BFT growth partitioned more N into herbage than for other forages, while legumes invested less N in crowns and roots than the grass and the forb.

It is important to mention that approximately 90% of the N consumed by cattle in pastures is excreted, mostly in urine, and returns to the grazing system (Whitehead et al., 1986). The high N concentration of each waste application results in losses through ammonia volatilization, denitrification and, potentially, through leaching, although leaching was minimal in the forage systems we considered. Calculated N losses due to volatilization of NH_3 derived mainly from N in the urine and amounted to 4 to 10% of the N added to these grazing systems as urine and feces, unlike values reported by previous

authors (Vallis, 1985; Whitehead et al., 1989) who found NH_3 volatilization amounted to 12-46% of the N applied from urea based on increased range of rates of N application to the soil. Small burnet is predicted to lose less N by volatilization due to HT increasing partitioning of N to feces (Waghorn et al., 1994; Barry and McNabb, 1999; Stewart et al., 2019). As noted by Haynes and Williams (1993), the lower the N losses, the more sustainable the system.

While the addition of peat moss to the soil meant that soil microbial activity was not constrained by low soil C, creation of new soil OM depends on a source of N that will not be lost through leaching or volatilization, even under frequent irrigation. A common fertilization rate for productive, well-managed grass pastures is 160 kg N ha^{-1}, equivalent to nearly 500 kg ha^{-1} over the 3 field seasons represented by this study. The organic and inorganic N in the manure added early in this study was in the range of 3000 kg N ha^{-1} for all four systems, and the final gain of approximately 6000-8500 kg ha^{-1} of new organic matter for MB and SB represented a 2-3 fold return on that investment. To the extent this N was mineralized, it appears to have been immobilized by the soil microbial biomass and accumulated as soil OM (Garret et al., 1992; Haynes and Williams, 1993; Jarvis, 1993).

5 CONCLUSIONS

Nitrogen is a valuable but economically and environmentally costly input in all agricultural ecosystems in the world. In this controlled environment study, the legumes BFT and CMV added 758 and 182 kg ha^{-1} fixed N, respectively, to their systems.

Another approximately 3000 kg ha^{-1} N was added as ruminant waste sourced from cattle fed the same forage species, although the partitioning of N to urine and feces differed among species. Leaching was negligible for all species, and soil organic N was approximately 2-3-fold return on the initial investment for MB and SB, respectively, over the course of the study, which comprised eight harvests, or roughly the equivalent of the initial three years of a perennial forage stand. While the two *non*-legume systems accumulated new soil organic N, the two legume systems did not, and the source of the added N is not known. The two waste applications that occurred after the first two harvests added between 383 and 1480 kg N ha^{-1} as urine, a readily available source of inorganic N. Volatilization was calculated as a function of urine application, and ranged from 124 to 277 kg N ha^{-1}. Organic N was added as feces and ranged from 1163 to 2556 kg N ha^{-1}, and plant components synthesized over the course of the study contained a total of between 1107 and 1851 kg N ha^{-1}. Perennial legumes invested less N in roots and crowns than the forb and grass, and BFT produced significantly more herbage DM and fixed more N$_2$ than CMV.

By the end of the study, soil inorganic N had increased nearly four-fold; data for individual species was not significantly different so the same value was used for the final inorganic N of all species. The accumulation of soil OM facilitated by perennial root proliferation and root pruning after grazing or harvest is a feature of the cultivation of perennial forages, which enhance soil quality and sequester C. Under increasingly erratic weather extremes caused by climate change, these valuable traits can protect the environment and increase the sustainability of grazing systems. The results of this study

can serve as a source of data for life cycle assessments of ruminant meat and milk

production systems.

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TABLES

TABLE 4-1 Mixed soil plus peat analysis determined by the USU Analytical Lab.

	Soil	Soil + Peat Moss
pH	7.2	6.1
ECe, dS m^{-1}	0.39	0.74
P, mg kg^{-1}	184.0	47.5
K, mg kg^{-1}	249.0	312.0
Organic matter, %	5.0	12.5

TABLE 4-2 Feces and urine concentrations of inorganic and organic N, $\delta^{15}N$, total carbon, and tannin from cattle fed hay of the four treatment species and used to fertilize forages of the same plant species.

	Ammonium (mg kg^{-1})	Nitrate (mg kg^{-1})	Organic N (g kg^{-1})	$\delta^{15}N$ (‰)	Total C (g kg^{-1})	Tannin (g kg^{-1})
			Feces			
BFT	1047.22	7.04	24.95	43.11	522.4	12.00
CMV	1128.62	9.88	27.06	19.94	450.9	10.30
MB	41.97	8.77	17.05	40.95	453.9	4.10
SB	725.58	6.24	31.67	55.63	452.3	12.50
			Urine			
BFT	46.59	1.26	74.15	0.31	172.4	
CMV	114.94	1.91	117.68	2.20	194.2	
MB	134.83	2.51	98.56	-0.57	255.9	
SB	241.73	3.20	31.76	2.71	207.8	

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TABLE 4-3 Total N, C and tannin (kg ha^{-1}) applied during the study as feces and urine from cattle fed hay of the same species.

	Ammonium	Nitrate	Organic N	Total C	Tannin
Feces					
BFT	69.91	0.005	1609	33,740	7750
CMV	75.84	0.007	1757	29,311	6696
MB	59.26	0.007	1163	32,455	2932
SB	3.12	0.005	2556	35,729	9874
Urine					
BFT	0.01	0.00	1300	3020	
CMV	0.01	0.00	1480	2439	
MB	0.02	0.00	989	2564	
SB	0.02	0.00	383	2485	

TABLE 4-4 Herbage DM (kg ha^{-1}) ± SEM removed at each harvest and their totals.

Harvest dates	BFT	CMV	MB	SB
1/19/2017	9618 (2229) [A]	9809 (2293) [A]	6911 (1624)	6720 (1561) [A]
3/2/2017	6178 (1433) [AB a]	5096 (1178) [B a]	5159 (1210) [a]	2102 (478) [B b]
4/19/2017	6783 (1592) [AB a]	1688 (382) [CD b]	5446 (1274) [a]	2357 (541) [B b]
5/26/2017	8981 (2102) [AB a]	2293 (541) [CD b]	3981 (924) [b]	4777 (1115) [AB ab]
7/6/2017	7930 (1847) [AB a]	2580 (605) [BCD c]	3312 (764) [bc]	6783 (1592) [A ab]
8/18/2017	7739 (1815) [AB a]	3535 (828) [BC b]	4140 (955) [ab]	7134 (1656) [A ab]
9/30/17	5159 (1210) [AB]	3217 (764) [BC]	2771 (637)	4554 (1051) [AB]
10/31/2017	3822 (892) [B a]	1497 (350) [D b]	3439 (796) [a]	4841 (1115) [AB a]
Total	58,121 (7293) [a]	31,051 (3917) [b]	37,389 (4713) [b]	40,446 (5096) [ab]

[A-D] LSmeans in columns with different uppercase letters differ at $P<0.05$. [a-c] LSmeans in rows with different lowercase letters differ at $P<0.05$. LSmeans based on 4 spatial replications.

TABLE 4-5 Herbage N concentration (g kg⁻¹) ± SEM at each harvest and species means.

Harvest dates	BFT	CMV	MB	SB
1/19/2017	24.5 (2.1) $^{B\ a}$	24.3 (2.1) $^{D\ a}$	12.9 (1.1) $^{B\ b}$	14.7 (1.3) $^{B\ b}$
3/2/2017	34.5 (3.0) $^{A\ a}$	28.4 (2.4) $^{BCD\ ab}$	24.9 (2.1) $^{A\ b}$	23.8 (2.1) $^{A\ b}$
4/19/2017	30.5 (2.6) $^{AB\ b}$	40.0 (3.4) $^{A\ a}$	26.0 (2.2) $^{A\ b}$	27.3 (2.4) $^{A\ b}$
5/26/2017	28.7 (2.5) $^{AB\ b}$	36.7 (3.2) $^{AB\ a}$	21.8 (1.9) $^{A\ c}$	28.4 (2.4) $^{A\ b}$
7/6/2017	25.7 (2.2) $^{B\ b}$	36.3 (3.1) $^{AB\ a}$	24.0 (2.1) $^{A\ b}$	24.6 (2.1) $^{A\ b}$
8/18/2017	30.1 (2.6) $^{AB\ ab}$	34.5 (3.0) $^{ABC\ a}$	24.3 (2.1) $^{A\ b}$	23.6 (2.0) $^{A\ b}$
9/30/17	32.7 (2.8) $^{AB\ ab}$	35.3 (3.0) $^{AB\ a}$	25.1 (2.2) $^{A\ c}$	25.8 (2.2) $^{A\ bc}$
10/31/2017	29.0 (2.5) $^{AB\ a}$	27.0 (2.3) $^{CD\ ab}$	24.0 (2.1) $^{A\ ab}$	22.3 (1.9) $^{A\ b}$
Mean	29.5	32.8	22.9	23.8

$^{A-D}$ LSmeans in columns with different uppercase letters differ at $P<0.05$. $^{a-c}$ LSmeans in rows with different lowercase letters differ at $P<0.05$. LSmeans based on 4 spatial replications.

TABLE 4-6 Herbage N (kg ha^{-1}) ± SEM at each harvest and species totals.

Harvest dates	BFT	CMV	MB	SB
1/19/2017	238 (24) [AB a]	247 (24) [A a]	91 (24) [b]	105 (24) [AB b]
3/2/2017	223 (31) [ABC a]	163 (31) [B a]	129 (31) [ab]	54 (31) [B b]
4/19/2017	228 (34) [ABC a]	72 (34) [CD b]	149 (34) [ab]	79 (34) [AB b]
5/26/2017	259 (17) [A a]	86 (17) [CD c]	90 (17) [bc]	141 (17) [A b]
7/6/2017	205 (23) [BC a]	95 (23) [BCD b]	92 (23) [b]	174 (23) [A a]
8/18/2017	238 (28) [AB a]	124 (28) [BC b]	113 (28) [b]	178 (28) [A ab]
9/30/17	205 (37) [ABC a]	115 (37) [BCD ab]	88 (37) [b]	123 (37) [AB ab]
10/31/2017	131 (23) [D a]	43 (23) [D b]	84 (23) [ab]	112 (23) [AB ab]
Total	1725 (141) [a]	945 (141) [b]	835 (141) [b]	965 (141) [b]

[A-D] LSmeans in columns with different uppercase letters differ at $P<0.05$. [a-c] LSmeans in rows with different lowercase letters differ at $P<0.05$. LSmeans based on 4 spatial replications.

TABLE 4-7 Irrigation water (L ha^{-1}) ± SEM added prior to each harvest and species totals.

Harvest dates	BFT	CMV	MB	SB
1/19/2017	7962 (637) [A]	7006 (637) [A]	8280 (637) [A]	7962 (637) [A]
3/2/2017	4459 (637) [AB]	3822 (318) [B]	5732 (637) [AB]	3822 (318) [B]
4/19/2017	4140 (955) [AB]	1274 (318) [C]	6688 (1911) [AB]	2866 (637) [B]
5/26/2017	6051 (955) [AB]	2229 (318) [C]	4777 (955) [BC]	5414 (955) [AB]
7/6/2017	6688 (1274) [A]	2229 (318) [BC]	4777 (955) [ABC]	8599 (1592) [A]
8/18/2017	6369 (1274) [A]	2866 (637) [BC]	5096 (955) [AB]	7006 (1274) [A]
9/30/17	7643 (2548) [A]	2866 (955) [BC]	1911 (637) [CD]	5096 (1592) [AB]
10/31/2017	2548 (637) [B]	1274 (318) [C]	1911 (637) [D]	2548 (637) [B]
Total	47,134 (4140) [a]	23,885 (1274) [b]	43,312 (4140) [a]	44,904 (4140) [a]

[A-D] LSmeans in columns with different uppercase letters differ at $P<0.05$. [a-b] LSmeans in rows with different lowercase letters differ at $P<0.05$. LSmeans based on 4 spatial replications.

TABLE 4-8 Ammonium, nitrate and their sum (inorganic N; g ha^{-1}) ± SEM in a 500 mL leaching fraction added prior to each harvest, totaled by harvest and at the end of the study. Manure was added one week after the first and second harvests.

Dates:	1/17/17	3/1/17	4/18/17	5/25/17	7/5/17	8/17/17	9/29/17	10/30/17	Total
					BFT				
NH_4^+	8 (9)	1 (1)	0 (1)	31 (40)	4 (6)	10 (10)	2 (2) [B]	18 (10) [A]	156 (68) [AB]
NO_3^-	6 (6)	1 (2)	4 (6)	101 (168)	6 (7)	22 (26)	1 (1)	5 (4)	374 (180) [A]
Total N	18 (20)	3 (3)	5 (7)	138 (220)	11 (13)	34 (36)	2 (3) [B]	23 (13)	577 (324)
					CMV				
NH_4^+	4 (5)	4 (5)	2 (2)	2 (3)	2 (2)	28 (28)	5 (6) [AB]	6 (3) [AB]	127 (56) [B]
NO_3^-	2 (2)	1 (1)	11 (14)	4 (7)	6 (7)	11 (13)	5 (6)	6 (5)	246 (18) [AB]
Total N	6 (7)	6 (7)	14 (19)	12 (20)	12 (14)	40 (41)	15 (19) [AB]	17 (10)	487 (274)
					MB				
NH_4^+	3 (3)	4 (5)	2 (3)	10 (13)	36 (47)	9 (9)	17 (21) [AB]	9 (5) [AB]	382 (167) [AB]
NO_3^-	2 (2)	3 (3)	1 (1)	4 (7)	11 (12)	2 (3)	3 (4)	1 (1)	91 (10) [B]
Total N	4 (5)	7 (8)	4 (5)	16 (26)	55 (68)	13 (13)	22 (27) [AB]	12 (7)	523 (294)
					SB				
NH_4^+	6 (6) [ab]	1 (2) [b]	11 (16) [ab]	1 (2) [b]	1 (1) [b]	8 (8) [ab]	208 (260) [A a]	3 (2) [B b]	588 (257) [A]
NO_3^-	3 (3)	1 (1)	26 (35)	3 (5)	0 (1)	5 (5)	26 (32)	1 (1)	223 (121) [AB]
Total N	9 (10) [ab]	2 (3) [b]	55 (76) [ab]	5 (8) [ab]	2 (2) [b]	17 (17) [ab]	276 (337) [A a]	4 (2) [b]	859 (483)

[A-B] LSmeans for a given variable within a column with different uppercase letters differ at *P*<0.05. [a-b] LSmeans within a row with different lowercase letters differ at *P*<0.05. LSmeans based on 4 spatial replications.

TABLE 4-9 Soil N and C concentrations (g kg^{-1}) ± SEM at destructive harvest.

	Species	Depth, cm			
		0-10	10-30	30-60	60-75
Organic N	BFT	4.6 (0.5) [a]	3.0 (0.3) [b]	2.9 (0.3) [b]	2.8 (0.3) [b]
	CMV	5.0 (0.5) [a]	2.7 (0.3) [b]	3.0 (0.3) [b]	2.9 (0.3) [b]
	MB	4.5 (0.5) [a]	2.7 (0.3) [b]	2.9 (0.3) [b]	2.9 (0.3) [b]
	SB	4.5 (0.5) [a]	2.6 (0.3) [b]	2.7 (0.3) [b]	2.6 (0.3) [b]
	Mean	4.7 (0.4) [a]	2.8 (0.3) [b]	2.9 (0.3) [b]	2.8 (0.3) [b]
Organic C	BFT	59.3 (5.0) [a]	41.6 (1.7) [b]	41.1 (1.9) [b]	40.8 (1.6) [B b]
	CMV	64.9 (5.0) [a]	36.9 (1.7) [c]	43.3 (1.9) [b]	43.2 (1.6) [AB b]
	MB	65.0 (5.0) [a]	39.0 (1.7) [c]	40.7 (1.9) [bc]	45.1 (1.6) [AB b]
	SB	63.1 (5.0) [a]	37.7 (1.7) [c]	42.1 (1.9) [bc]	46.5 (1.6) [A b]
	Mean	63.1 (5.0)	38.8 (1.7)	41.8 (1.9)	43.9 (1.6)

[A-B] LSmeans for a given variable in columns with different uppercase letters differ at $P<0.05$. [a-c] LSmeans in rows with different lowercase letters differ at $P<0.05$. LSmeans based on 4 spatial replications.

TABLE 4-10 Soil organic N and C (kg ha^{-1}) ± SEM and their totals at destructive harvest.

Soil Properties	Species	Depth, cm				
		0-10	10-30	30-60	60-75	Total
Organic N,	BFT	5983 (740) [c]	7608 (874) [b]	11,384 (1243) [a]	5360 (757) [c]	26,033 (1113) [B]
(± SEM)	CMV	6435 (740) [b]	7031 (874) [b]	11,416 (1243) [a]	5575 (757) [b]	24,243 (1113) [B]
	MB	5782 (740) [b]	6943 (874) [b]	11,282 (1243) [a]	5646 (757) [b]	32,414 (1113) [A]
	SB	5886 (740) [bc]	6779 (874) [b]	10,315 (1243) [a]	5119 (757) [c]	35,853 (1113) [A]
Organic C,	BFT	77,041 (6290) [c]	108,095 (5389) [b]	160,095 (7410) [a]	79,511 (3540) [c]	452,733 (9122) [A]
(± SEM)	CMV	84,354 (6290) [b]	95,810 (5389) [b]	168,724 (7410) [a]	84,240 (3540) [b]	427,552 (8615) [AB]
	MB	84,549 (6290) [b]	101,270 (5389) [b]	158,779 (7410) [a]	88,018 (3540) [b]	416,220 (8387) [B]
	SB	81,981 (6290) [b]	98,053 (5389) [b]	164,092 (7410) [a]	90,724 (3540) [b]	427,781 (8619) [AB]

[A-B] LSmeans for a given variable in columns with different uppercase letters differ at $P<0.05$. [a-c] LSmeans in rows with different lowercase letters differ at $P<0.05$. LSmeans based on 4 spatial replications.

TABLE 4-11 Soil inorganic N concentrations (KCl-extractable ammonium and nitrate; mg N kg^{-1} soil) ± SEM and their means at destructive harvest.

Soil	Species	Depth, cm			
		0-10	10-30	30-60	60-75
NH_4^+	BFT	2.4 (1.1) [b]	1.8 (1.2) [ab]	5.6 (4.9) [ab]	15.6 (12.9) [a]
	CMV	6.9 (3.1) [b]	27.8 (19.5) [ab]	31.4 (27.2) [ab]	27.4 (22.7) [a]
	MB	5.0 (2.2) [b]	8.8 (6.2) [ab]	16.0 (13.9) [ab]	34.7 (28.8) [a]
	SB	1.8 (0.8) [b]	3.3 (2.3) [ab]	0.4 (0.4) [ab]	10.7 (8.8) [a]
	Mean	3.7 (1.1) [b]	7.2 (2.9) [ab]	8.8 (4.1) [ab]	20.2 (9.2) [a]
NO_3^-	BFT	7.6 (4.8) [a]	1.6 (1.3) [b]	0.7 (0.5) [bc]	1.4 (1.0) [c]
	CMV	23.4 (14.7) [a]	1.0 (0.8) [b]	0.4 (0.3) [bc]	-0.2 (-0.2) [c]
	MB	4.6 (2.9) [a]	5.6 (4.4) [b]	3.6 (2.7) [bc]	-0.5 (-0.3) [c]
	SB	15.3 (9.7) [a]	4.6 (3.6) [b]	3.5 (2.6) [bc]	2.1 (1.5) [c]
	Mean	12.7 (8.0) [a]	3.2 (2.5) [b]	2.0 (1.5) [bc]	0.7 (0.5) [c]

[a-c] LSmeans in rows with different lowercase letters differ at $P<0.05$. LSmeans based on 4 spatial replications.

TABLE 4-12 Soil inorganic N (KCl-extractable ammonium and nitrate; kg N ha^{-1}) ± SEM and their totals at destructive harvest.

Soil	Species	Depth, cm				
		0-10	10-30	30-60	60-75	Total
KCl Extractable						
NH$_4^+$	BFT	5 (1)	19 (7)	45 (26)	37 (16)	320 (111)
	CMV	16 (3) [b]	82 (29) [a]	130 (73) [a]	57 (25) [ab]	197 (111)
	MB	7 (1) [b]	28 (10) [ab]	69 (39) [ab]	89 (39) [a]	307 (111)
	SB	2 (0)	11 (4)	11 (6)	32 (14)	141 (111)
	Mean					241 (111)
NO$_3^-$	BFT	13 (3)	13 (4)	4 (1)	4 (0)	53 (25)
	CMV	61 (14) [a]	13 (4) [b]	11 (2) [b]	0 (0) [b]	74 (35)
	MB	7 (2)	18 (5)	18 (4)	1 (0)	23 (13)
	SB	20 (5)	12 (3)	17 (3)	7 (1)	69 (32)
	Mean					55 (27)

[a-b] LSmeans in rows with different lowercase letters differ at $P<0.05$. LSmeans based on 4 spatial replications.

TABLE 4-13 Root length density (RLD; cm cm^{-3}) ± SEM and root surface density (RSD; cm^2 cm^{-3}) ± SEM at destructive harvest.

		Depth, cm			
	Species	0-10	10-30	30-60	60-75
RLD	BFT	54.7 (9.2) $^{AB\ a}$	23.3 (9.2) $^{B\ b}$	43.3 (9.2) ^{ab}	44.9 (9.2) $^{AB\ ab}$
	CMV	47.0 (9.2) ^{B}	31.6 (9.2) ^{AB}	51.7 (9.2)	56.4 (9.2) ^{AB}
	MB	75.9 (9.2) $^{A\ a}$	32.0 (9.2) $^{AB\ c}$	46.5 (9.2) ^{bc}	70.1 (9.2) $^{A\ ab}$
	SB	75.9 (9.2) $^{A\ a}$	55.3 (9.2) $^{A\ ab}$	57.6 (9.2) ^{ab}	36.7 (9.2) $^{B\ b}$
RSD	BFT	7.1 (1.1) ^{a}	2.8 (0.9) $^{B\ b}$	5.0 (1.1) ^{ab}	4.9 (1.2) $^{AB\ ab}$
	CMV	6.1 (1.1)	3.8 (0.9) ^{AB}	6.5 (1.1)	6.4 (1.2) ^{AB}
	MB	8.7 (1.1) ^{a}	3.7 (0.9) $^{AB\ b}$	5.4 (1.1) ^{ab}	7.7 (1.2) $^{A\ a}$
	SB	9.0 (1.1) ^{a}	6.3 (0.9) $^{A\ ab}$	7.1 (1.1) ^{ab}	3.5 (1.2) $^{B\ b}$

$^{A-B}$ LSmeans for a given variable in columns with different uppercases letters differ at $P<0.05$. $^{a-c}$ LSmeans in rows with different lowercases letters differ at $P<0.05$. LSmeans based on 4 spatial replications.

TABLE 4-14 Total root length (Mm ha^{-1}) ± SEM and root surface area (km^2 ha^{-1}) ± SEM and their totals at destructive harvest.

	Species	0-10	10-30	30-60	60-75	Total
				Depth, cm		
Root length	BFT	613 (133) [b]	523 (200) [b]	1456 (345) [a]	755 (214) [ab]	3177 (398)
	CMV	527 (133) [b]	708 (200) [b]	1739 (345) [a]	948 (214) [ab]	3831 (480)
	MB	851 (133) [ab]	719 (200) [b]	1565 (345) [a]	1178 (214) [ab]	4275 (536)
	SB	851 (133) [bc]	1240 (200) [ab]	1938 (345) [a]	618 (214) [c]	4608 (577)
Root surface area	BFT	0.80 (0.16)	0.64 (0.24)	1.68 (0.42)	0.82 (0.23)	3.73 (0.47)
	CMV	0.69 (0.16) [b]	0.86 (0.24) [b]	2.18 (0.42) [a]	1.07 (0.23) [b]	4.72 (0.59)
	MB	0.98 (0.16)	0.82 (0.24)	1.81 (0.42)	1.30 (0.23)	4.84 (0.60)
	SB	1.01 (0.16) [bc]	1.42 (0.24) [ab]	2.39 (0.42) [a]	0.59 (0.23) [c]	5.38 (0.67)

[a-c] LSmeans in rows with different lowercases letters differ (*P*<0.05). LSmeans based on 4 spatial replications.

TABLE 4-15 Root dry matter concentrations (mg root cm^{-3} of soil) ($\pm$ SEM) and their means by soil layer and species at destructive harvest.

		Depth, cm				
	Species	0-10	10-30	30-60	60-75	Mean
Root DM	BFT	0.7 (0.1) $^{C\,a}$	0.6 (0.1) $^{B\,a}$	0.2 (0.0) ^{ab}	0.0 (0.0) $^{B\,b}$	0.4 (0.1)
	CMV	0.8 (0.2) $^{C\,a}$	0.3 (0.0) $^{B\,ab}$	0.2 (0.0) ^{ab}	0.0 (0.0) $^{B\,b}$	0.3 (0.1)
	MB	7.7 (1.5) $^{A\,a}$	0.7 (0.1) $^{B\,b}$	0.2 (0.0) ^{c}	0.2 (0.0) $^{A\,c}$	2.2 (0.4)
	SB	2.5 (0.5) $^{B\,a}$	1.8 (0.3) $^{A\,a}$	0.2 (0.0) ^{b}	0.0 (0.0) $^{B\,c}$	1.1 (0.2)
Mean		2.9 (0.6)	0.8 (0.1)	0.2 (0.0)	0.1 (0.0)	

$^{A\text{-}C}$ LSmeans in columns with different uppercases letters differ at $P<0.05$. $^{a\text{-}c}$ LSmeans in rows with different lowercases letters differ at $P<0.05$. LSmeans based on 4 spatial replications.

TABLE 4-16 Root dry matter (kg ha^{-1}) ± SEM and their totals at destructive harvest.

	Species	Depth, cm				
		0-10	10-30	30-60	60-75	Total
Root DM	BFT	714 (230) [a]	1020 (464) [a]	367 (238) [a]	36 (22) [b]	3268 (1630) [BC]
	CMV	735 (237)	407 (185)	215 (139)	105 (110)	2195 (1630) [C]
	MB	8491 (2740) [a]	1465 (665) [b]	684 (509) [b]	435 (268) [b]	12,210 (1630) [A]
	SB	2685 (866) [ab]	3985 (1811) [a]	710 (460) [b]	56 (42) [c]	7894 (1630) [AB]

[A-C] LSmeans in columns with different uppercases letters differ at $P<0.05$. [a-c] LSmeans in rows with different lowercases letters differ at $P<0.05$. LSmeans based on 4 spatial replications.

TABLE 4-17 Root N and C concentrations (g kg^{-1} root DM) ± SEM and their means at destructive harvest.

Root	Species	0-10	10-30	30-60	60-75	Mean
				Depth, cm		
Nitrogen	BFT	12.2 (1.6) [b]	14.9 (1.9) [ab]	15.9 (0.7) [A ab]	15.8 (1.3) [A a]	14.6 (1.4)
	CMV	14.9 (1.8) [a]	13.0 (2.1) [ab]	10.0 (0.7) [C b]	ND	12.6 (1.5)
	MB	11.0 (1.6) [a]	10.2 (1.9) [ab]	7.1 (0.7) [D bc]	6.5 (1.2) [B c]	8.7 (1.4)
	SB	9.7 (1.6)	10.7 (1.9)	10.9 (0.7) [B]	ND	10.4 (1.4)
Carbon	BFT	400.5 (9.7) [A]	403.0 (9.9) [A]	411.5 (9.7) [A]	ND	405.0 (8.6) [A]
	CMV	409.8 (10.6) [A]	409.4 (10.0) [A]	405.4 (9.7) [A]	ND	408.2 (8.6) [A]
	MB	372.9 (9.7) [B]	379.8 (9.3) [B]	379.8 (8.3) [B]	385.5 (7.2)	379.5 (7.7) [B]
	SB	401.9 (9.7) [A]	412.4 (9.3) [A]	407.1 (8.7) [A]	ND	407.1 (8.3) [A]

[A-D] LSmeans for a given variable in columns with different uppercases letters differ at $P<0.05$. [a-c] LSmeans in rows with different lowercases letters differ at $P<0.05$. LSmeans based on 4 spatial replications.

TABLE 4-18 Total root organic N and C (kg ha^{-1}) ± SEM and their totals at destructive harvest.

Root	Species	Depth, cm				
		0-10	10-30	30-60	60-75	Total
Organic N	BFT	8 (2) [a]	15 (7) [a]	8 (3) [a]	2 (1) [b]	48 (20) [B]
	CMV	16 (5)	7 (4)	10 (4)	ND	28 (20) [B]
	MB	93 (25) [a]	15 (7) [b]	5 (2) [c]	4 (1) [c]	127 (20) [A]
	SB	26 (7) [a]	42 (19) [a]	8 (2) [b]	ND	78 (20) [AB]
Organic C	BFT	286 (73)	583 (249)	334 (156)	ND	1206 (593) [BC]
	CMV	511 (146)	304 (130)	295 (139)	ND	847 (593) [C]
	MB	3158 (806) [a]	556 (209) [b]	298 (117) [b]	258 (41) [b]	4491 (593) [A]
	SB	1078 (275) [a]	1642 (618) [a]	311 (122) [b]	ND	3119 (593) [AB]

[A-C] LSmeans for a given variable in columns with different uppercases letters differ at *P*<0.05. [a-c] LSmeans in rows with different lowercases letters differ at *P*<0.05. LSmeans based on 4 spatial replications.

TABLE 4-19 Values of $\delta^{15}N$ ($\pm$ SEM) for herbage obtained by the ^{15}N natural abundance method, along with the proportion of legume nitrogen derived from N_2 fixation (P_{fix}) and total N_2 fixed at the first, middle and last harvest during the study. Isotopic fractionation of the same legumes grown from seed in sand culture without external N was used to calculate P_{fix}.

Species	$\delta^{15}N$ (‰)	P_{fix} (%)	N_2 fixed (kg ha^{-1})
	January 2017		
BFT	7.84 (2.12) [b]	51.7	124
CMV	20.42 (6.82) [ab]	2.0	6
MB	33.92 (6.82) [a]		
SB	20.87 (6.82) [a]		
	May 2017		
BFT	10.97 (2.12)	10.5	29
CMV	14.71 (6.82)	0	0
MB	12.05 (6.82)		
SB	12.76 (6.82)		
	October 2017		
BFT	-1.49 (2.12)	83.3	118
CMV	2.90 (6.82)	69.6	32
MB	8.02 (6.82)		
SB	12.62 (6.82)		

[a-b] LSmeans in columns, within a date, with different letters differ ($P<0.05$).
LSmeans based on 4 spatial replications (blocks).
$\delta^{15}N$ = ^{15}N natural abundance; P_{fix} = proportion of BFT and CMV derived from N_2 fixation.

TABLE 4-20 Herbage biological nitrogen fixation (BNF, kg ha^{-1}) ± SEM in legumes at each harvest and their totals. Calculated P_{fix} for the three dates in Table 4-19 were used to create quadratic equations of P_{fix} as a function of date for each legume to predict isotopic discrimination and BNF for all harvest dates.

Harvest dates	BFT	CMV
1/19/2017	123 (5) [A a]	5 (5) [C b]
3/2/2017	85 (7) [AB a]	2 (7) [C b]
4/19/2017	52 (13) [BC a]	0 (13) [C b]
5/26/2017	27 (5) [C a]	0 (5) [C b]
7/6/2017	61 (3) [AB a]	17 (3) [AB b]
8/18/2017	117 (10) [A a]	46 (10) [A b]
9/30/17	142 (29) [A a]	64 (29) [A b]
10/31/2017	109 (23) [AB a]	30 (23) [A b]
Total	690 (104) [a]	164 (2) [b]

[A-C] LSmeans in columns with different uppercases letters differ at $P<0.05$.
[a-b] LSmeans in rows with different lowercase letters differ at $P<0.05$.
LSmeans based on 4 spatial replications.

TABLE 4-21 Dry matter (kg ha^{-1}), N concentrations (g kg^{-1} DM) and total N (kg ha^{-1}) with totals, ± SEM, in plant components at destructive harvest.

	BFT	CMV	MB	SB
		DM		
Herbage	3894 (956) [B ab]	1437 (398) [B b]	3435 (852) [B ab]	4809 (1163) [B a]
Crown	6644 (1580) [A b]	3712 (914) [A c]	11,603 (2704) [A a]	14,351 (3328) [A a]
Roots	3268 (1630) [B bc]	2195 (1630) [B c]	12,210 (1630) [A a]	7894 (1630) [B ab]
Total	15,019 (3464) [b]	7729 (3464) [b]	27,719 (3464) [a]	27,695 (3464) [a]
		N concentration		
Herbage	29.03 (3.43) [A]	27.04 (3.20) [A]	23.98 (2.85) [A]	22.28 (2.66) [A]
Crown	11.31 (1.41) [B b]	15.18 (1.85) [B a]	12.17 (1.50) [B ab]	11.71 (1.45) [B ab]
Roots	14.60 (1.40) [B]	12.60 (1.50) [B]	8.70 (1.40) [C]	10.40 (1.40) [B]
		Total N		
Herbage	124 (13) [A a]	41 (11) [AB b]	83 (12) [a]	110 (13) [AB a]
Crown	78 (12) [A b]	59 (11) [A b]	145 (14) [a]	174 (14) [A a]
Roots	48 (20) [B b]	28 (20) [B b]	127 (20) [a]	78 (20) [B ab]
Total	233 (56) [ab]	125 (21) [b]	347 (50) [a]	357 (48) [a]

[A-C] LSmeans for a given variable in columns with different uppercases letters differ at $P<0.05$. [a-c] LSmeans in rows with different lowercase letters differ at $P<0.10$. LSmeans based on 4 spatial replications.

TABLE 4-22 Accumulation of symbiotically fixed N (kg ha^{-1}) ± SEM in legume components at destructive harvest. Calculated P_{fix} for the three dates in Table 4-19 were used to create quadratic equations of P_{fix} as a function of date, and crown and root values were based on the mean rate of fixation determined for herbage DM for the eight 2017 harvests dates.

	BFT	CMV
Herbage	91 (32) [A a]	27 (6) [A b]
Crown	29 (4) [B a]	10 (3) [B b]
Roots	39 (17) [AB a]	8 (4) [B b]
Total	166 (48) [a]	48 (10) [b]

[A-B] LSmeans in columns with different uppercases letters differ at *P*<0.05.
[a-b] LSmeans in rows with different lowercase letters differ at *P*<0.10.
LSmeans based on 4 spatial replications.

TABLE 4-23 Nitrogen balance (kg ha^{-1}) at 75 cm soil depth ± SEM for the study period.

	BFT	CMV	MB	SB	
N Sources					
Initial soil organic N	25,953	25,953	25,953	25,953	
Initial soil inorganic N	79	79	79	79	
N$_2$ fixation	758 (125) [a]	182 (9) [b]	0	0	*P<0.0001*
Feces N	1609	1757	1163	2556	
Urine N	1300	1480	989	383	
System N Sources	*29,700 (125)*	*29,452 (9)*	*28,185*	*28,972*	
N Sinks					
Final soil organic N	26,033 (1113) [b]	24,243 (1113) [b]	32,414 (1113) [a]	35,853 (1113) [a]	*P<0.0001*
Final soil inorganic N	296 (138)	296 (138)	296 (138)	296 (138)	ND
Leached inorganic N	1 (0)	1 (0)	1 (0)	1 (0)	*P=0.8922*
Volatilization (estimated)	245	277	213	124	
Total herbage N	1725 (141) [a]	945 (141) [b]	835 (141) [b]	965 (141) [b]	*P=0.0027*
Crown N	78 (12) [b]	59 (11) [b]	145 (14) [a]	174 (14) [a]	*P=0.0019*
Root N	48 (20) [b]	28 (20) [b]	127 (20) [a]	78 (20) [ab]	*P=0.0216*
System N Sinks	*28,426 (1424)*	*25,849 (1423)*	*34,031(1426)*	*37,491 (1426)*	
Newly Synthesized N	*-1274*	*-3602*	*5846*	*8519*	

[a-b] LSmeans in rows with different lowercase letters differ at *P*<0.05. LSmeans based on 4 spatial replications. Values without SEMs are from a single sample.
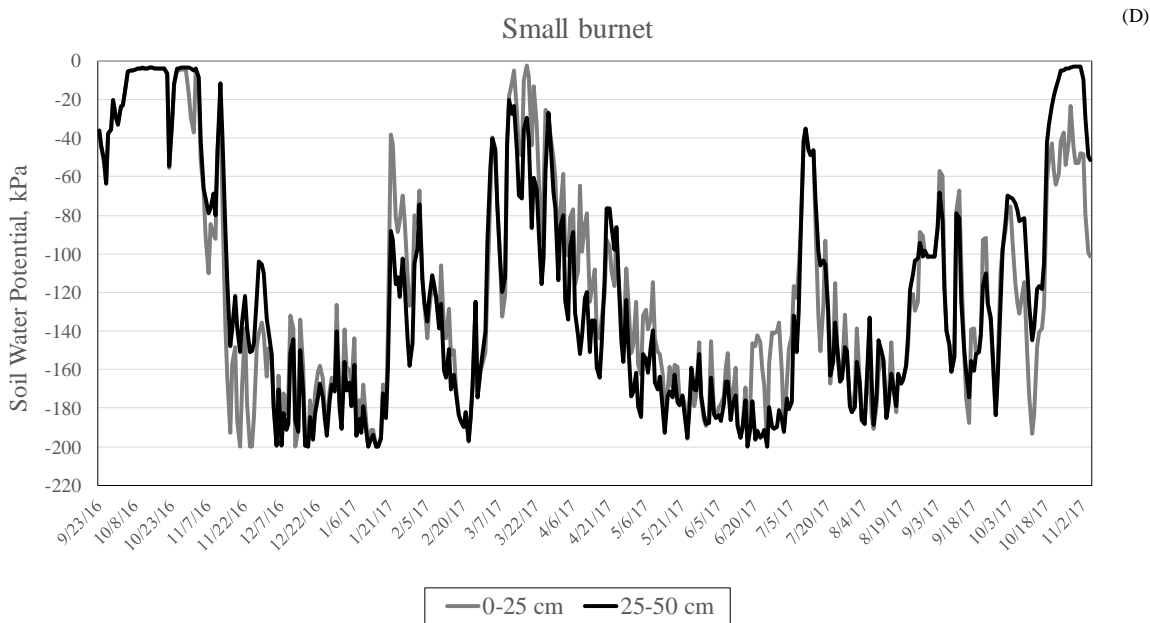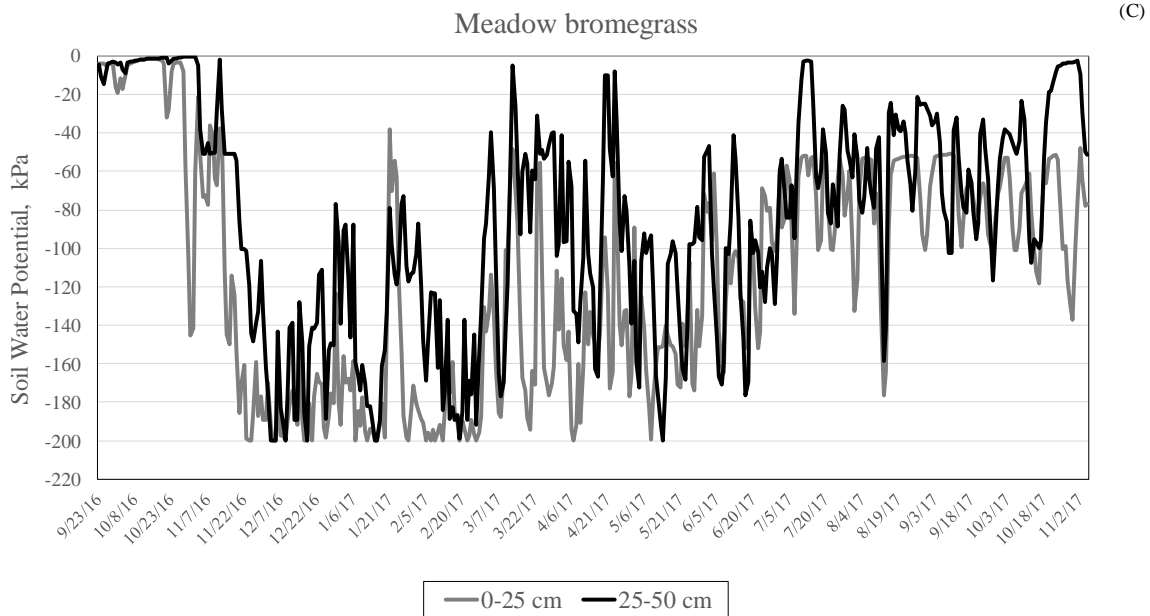
FIGURES

Birdsfoot trefoil

Cicer milkvetch

(C)

(D)
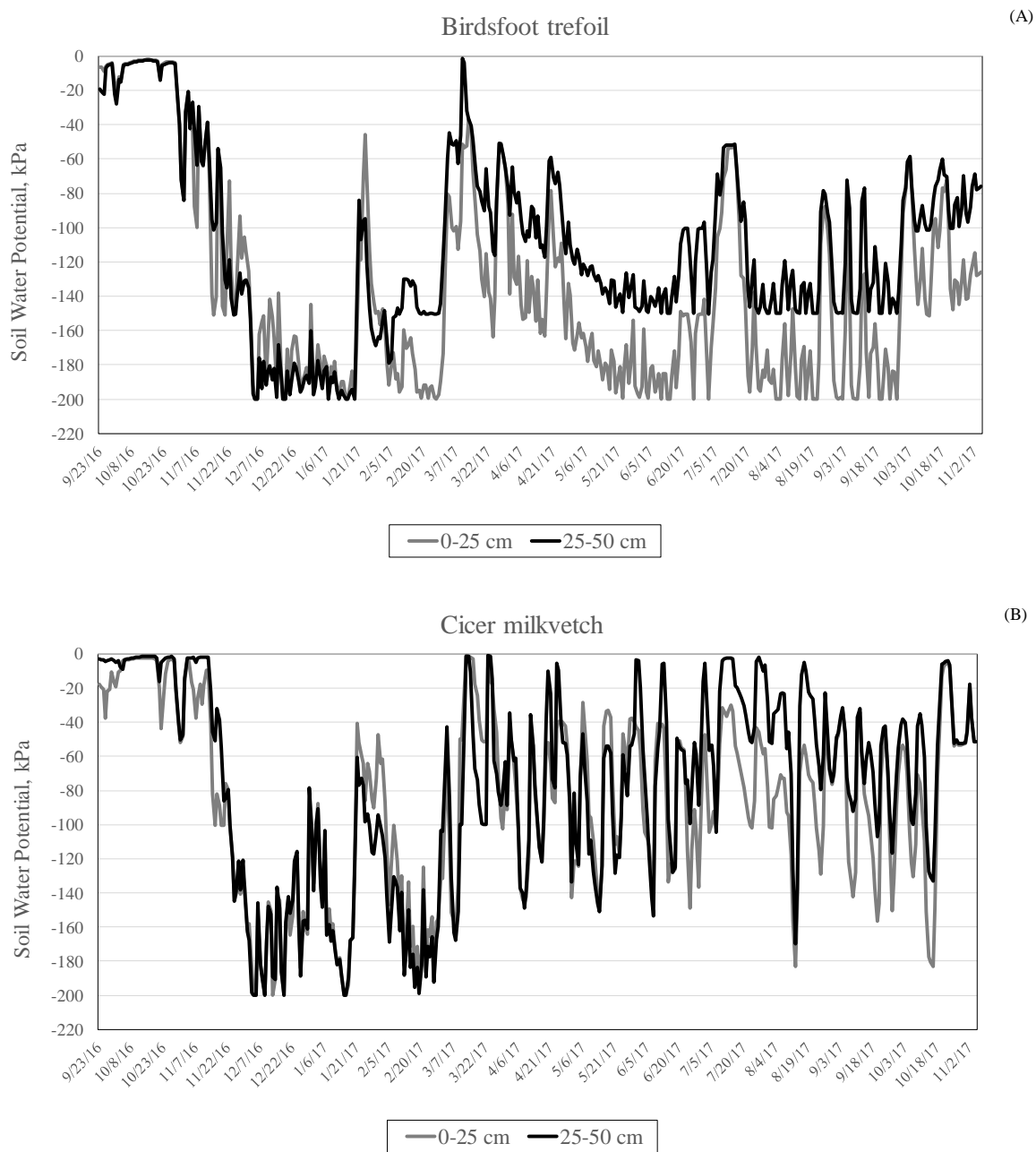
FIGURE 4-1 Mean soil water potential (kPa) of four replicate columns of each species at
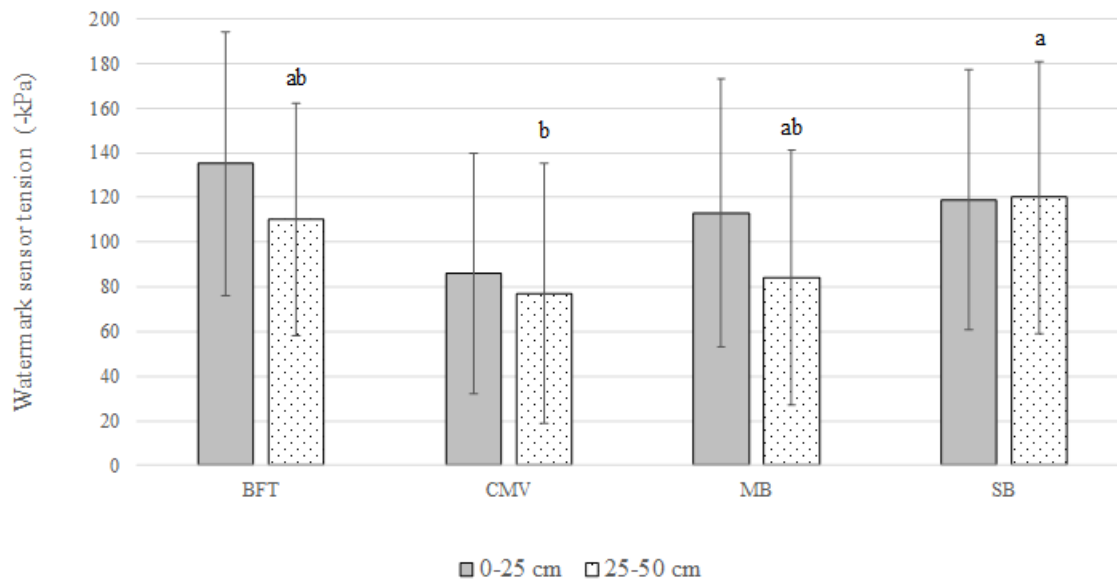
two depths (gray, 0-25 cm; black, 25-50 cm).

FIGURE 4-2 Mean soil tension ± SEM at two depths (gray, 0-25 cm; stippled, 25-50 cm) of four replicate columns of each forage species at two depths. Vertical bars represent standard error of the mean (SEM). Different lowercases above the bars indicate a significant difference among species within soil depth ($P<0.05$).
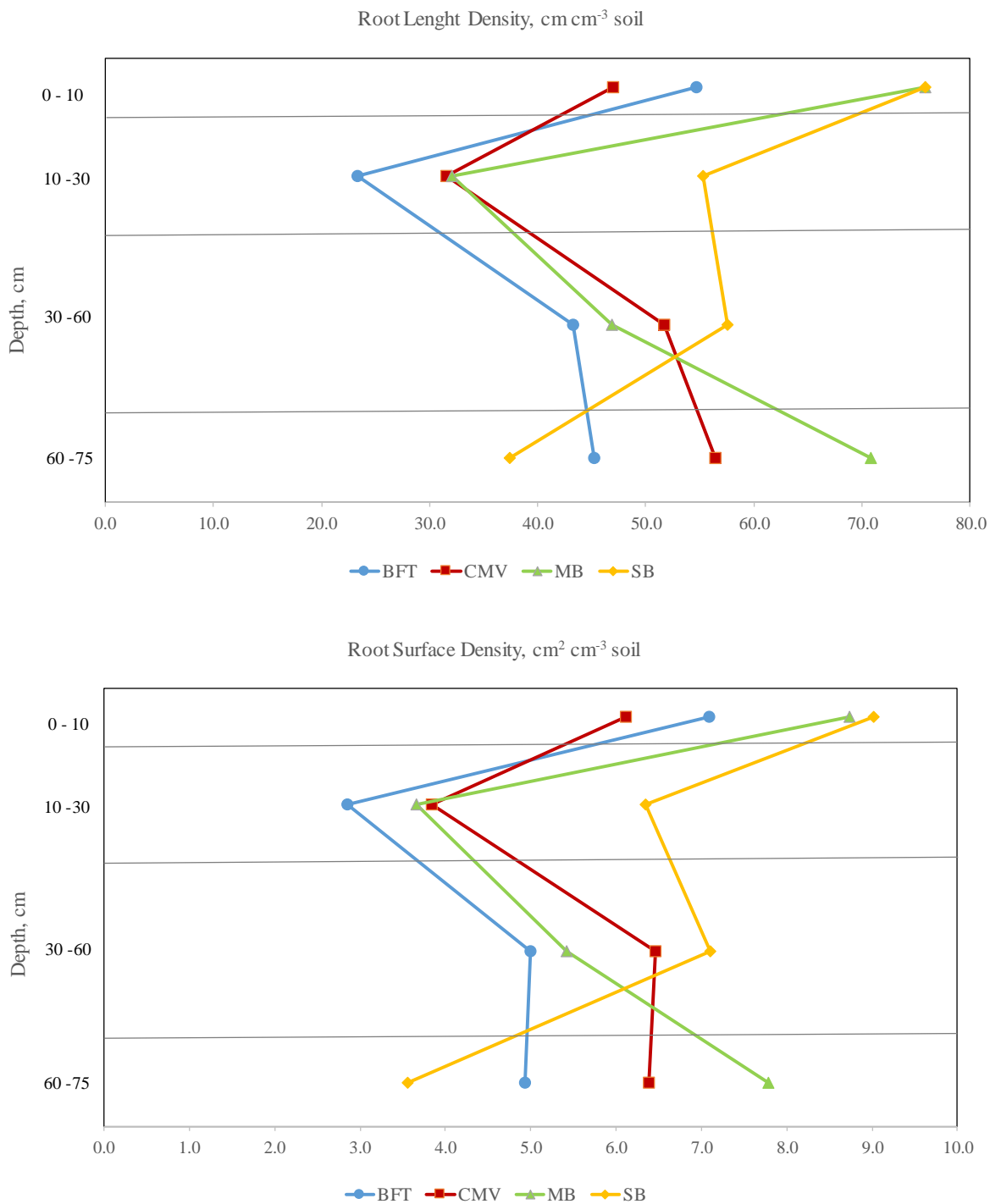
FIGURE 4-3 Root length density (RLD, cm root length cm^{-3} soil) and root surface density (RSD, cm^2 root surface area cm^{-3} soil) at destructive harvest.
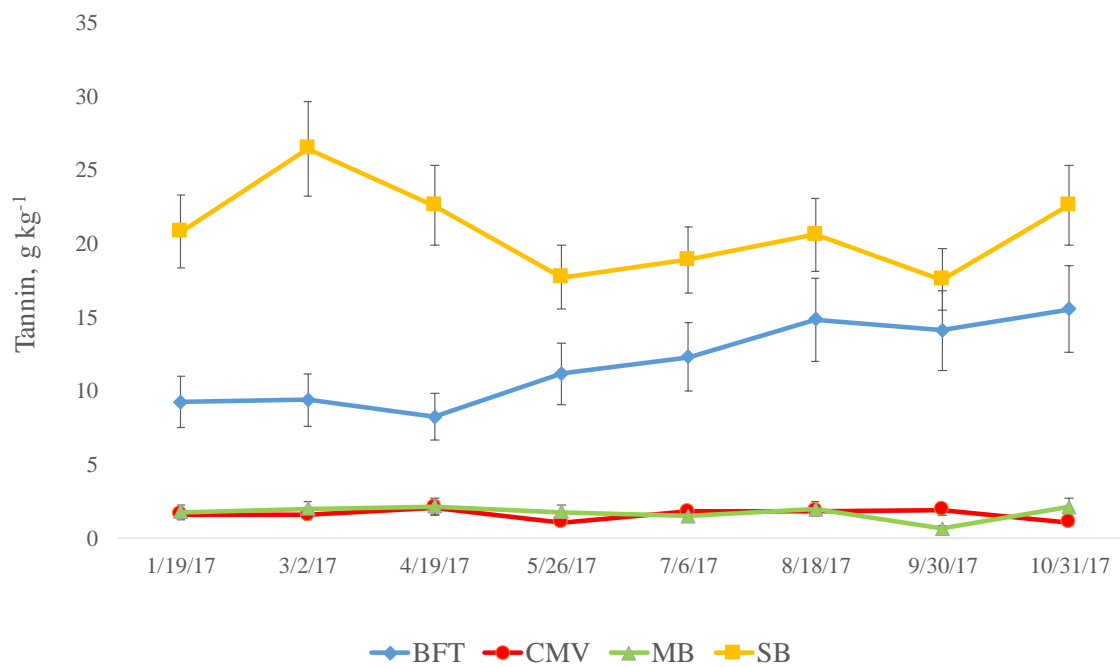
FIGURE 4-4 Herbage tannin concentrations (g kg^{-1}). LSmeans based on 4 spatial replications.
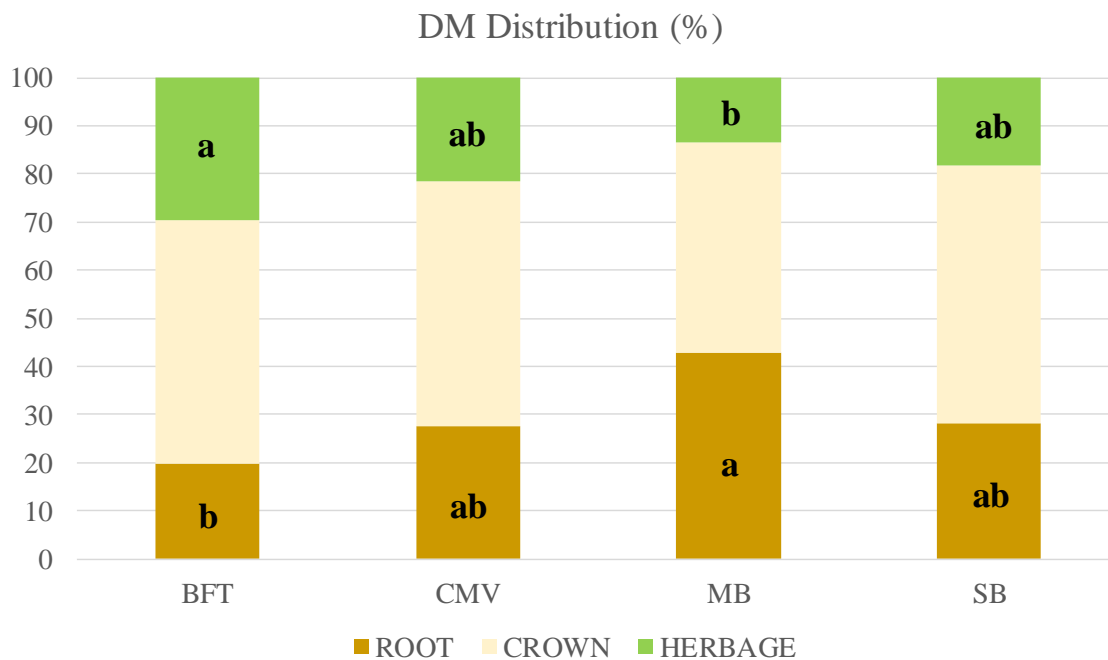
FIGURE 4-5 Forage component DM proportions at destructive harvest. Mean

separations of herbage and root DM based on LSmeans of 4 spatial replications; crown
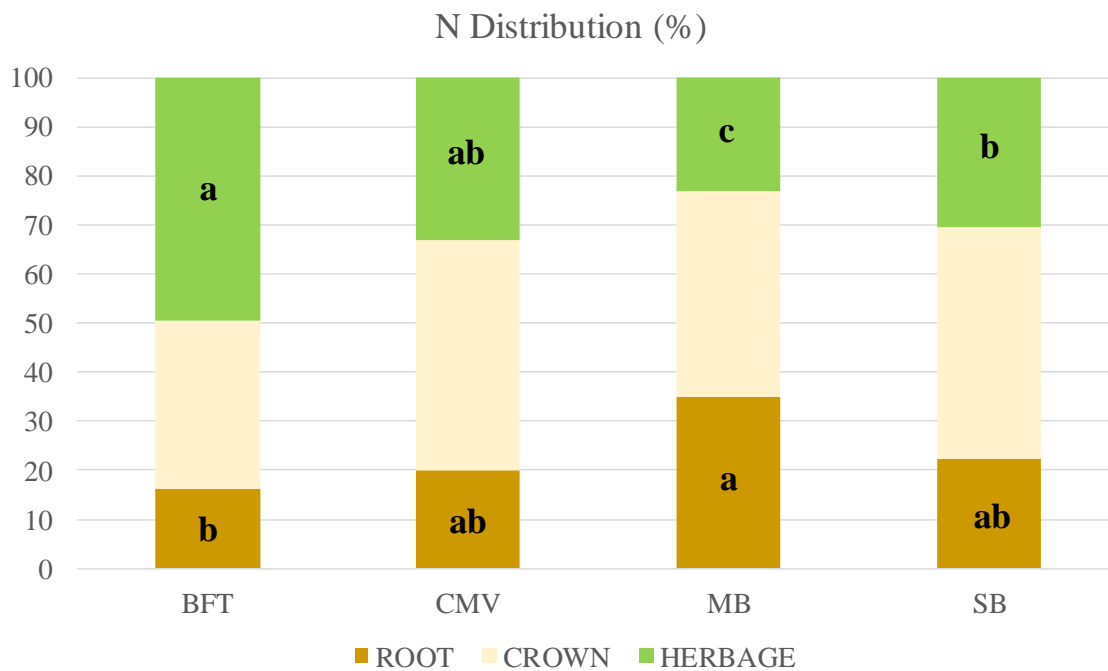
DM did not differ.

FIGURE 4-6 Forage component N distribution at destructive harvest. Mean separations

of herbage and root N based on LSmeans of 4 spatial replications; crown N did not differ.

CHAPTER V

SUMMARY AND CONCLUSIONS

My research demonstrated that the incorporation into beef production systems of alternative perennial legume pastures such as birdsfoot trefoil (*Lotus corniculatus* L.; BFT) and cicer milkvetch (*Astragalus cicer* L.; CMV) is beneficial relative to grass pastures such as meadow bromegrass (*Bromus biebersteinii Roem. & Schult*; MB). These legumes fix their own nitrogen (N) from atmospheric N_2, they are non-bloating and more digestible due to greater crude protein, greater non-fibrous carbohydrates, and lower fiber contents, resulted in greater dry matter intake. These characteristics mean that these legumes produce greater beef average daily gains per ha than grass pastures.

Birdsfoot trefoil is a tannin-containing legume, and tannin can lead to additional benefits for ruminants and the environment. Ruminants consuming condensed tannins (CT) in moderate doses (less than 5% of dry matter) have reduced internal parasite loads, more efficient utilization of ruminal protein resulting in greater fecal N to urinary N ratios that reduce soil N losses. The meat produced from ruminants grazing non-bloating legume pastures is more tender and juicier than the meat from grass-fed cattle, and the current research demonstrated that cattle grazing both legumes emitted less enteric methane (CH_4) than cattle on grass pastures.

Under field conditions (Chapter II) we did not detect a clear effect of CT lessening enteric CH_4 emissions, probably because the CT concentration of BFT is limited. However, we were able to conclude that the reductions measured in enteric CH_4

emissions were due to the greater feed quality of the two legumes which resulted in a significant reduction of the C footprint of beef production. Greater forage quality increased dry matter intake which is recognized as the main factor affecting the CH_4 footprint of beef production.

Both tannins and lignin may influence fermentation kinetics of forages through a negative effect on cellulose digestion. In an *in vitro* study (Chapter III) we found that hydrolysable tannins (HT) in small burnet (*Sanguisorba minor* Scop.; SB) and condensed tannins (CT) in sainfoin (*Onobrichis viciifolia* Scop.; SF) had reduced rates of ruminal colonization. Gas production early in the fermentation process along with reduced cumulative gas production can predict voluntary dry matter intake of forages by ruminants. Based on our research, the greatest whole plant dry matter intake would be expected for forage legumes due to their higher fermentation rates at the beginning of the incubation process (CMV and SF) or shorter half-time to maximum asymptotic gas production [alfalfa (*Medicago sativa* L.; ALF) and BFT], resulting in a lower total gas production for all legumes, faster rates of passage and reduced rumen fill. Legume rate of passage is also aided by their morphology, with short, heavily lignified veins in leaves.

Grasses such as MB, with greater fiber digestibility than other introduced, cool-season grasses, might be good pasture species for sequestration of atmospheric C due to greater root growth and rooting density than perennial legumes. Grass root turnover resulted in greater root C and N than legumes (Chapter II and IV), ultimately affecting the rhizosphere population. Microbial activity is enhanced through both root exudation and optimized C-N balance. Forages with greater root mass such as MB are known to

improve soil aggregation, and are generally associated with less NO_3^- leaching, as was detected in my study.

Decomposition and subsequent mineralization of dead plant parts release N as inorganic N. These rates are high for legume systems due to a low C:N ratio of the litter, inducing increased activity of the microbial community and faster decomposition and mineralization rates. Furthermore, manure in grazing systems is considered a source of organic N that supports greater diversity of soil microbes and improved soil quality and sustainability. When manure comes from animals consuming tannin-containing forages, soil properties may be further enhanced since tannins are known to improve soil function in beef production systems. Research carried out in a controlled environment (Chapter IV) demonstrated that tanniferous species such as BFT and small burnet (SB) tended to bind the N in the soil, thus reducing NO_3^- loss (BFT) while increasing soil organic C (BFT and SB) and root C (BFT and SB), where C availability to soil microbes drives N-cycling processes (N mineralization and immobilization rates), that in turn regulate N retention or N losses (denitrification, leaching and volatilization). Tannins can enhance soil quality, and improved nutrient cycling by these species results in greater potential for N and C sequestration, increasing the sustainability of these grazing production systems. Tannin-containing forages such as BFT and SB reduce the release rate of NO_3^- and N_2O from pasture systems and synchronize N demand by the plants with N mineralization in soil, reducing N losses and increasing the environment sustainability of pastures containing tanniferous legumes.

In this research, tannins did not negatively impact N_2 fixation rates, since BFT fixed the same amount of N than CMV (Chapter II) or 3.5 times more total N than CMV (Chapter IV). The rate of N_2 fixation, likely through reduced nodulation, was affected by elevated soil mineral N from manure, causing legumes to shut off fixation and use available soil N. Greater total N-fixed in BFT across the study was probably a consequence of a greater genetic ability to produce herbage dry matter, which required more N (Chapter IV).

Nitrogen is the nutrient most limiting to crop yield because it is needed for the enzymes that carry out primary metabolism, such as photosynthesis and respiration. Excess environmental N, however, can reduce air quality, contaminate ground water, contribute to eutrophication and global warming. Construction of N balances from different simulated grazing systems under controlled environmental conditions (Chapter IV) gave new information on the contribution of forages and grazing ruminants to the accumulation of soil organic N without increasing N losses that would pollute the environment, because leaching of both NO_3^- and NH_4^+ were minimal. Small burnet had elevated non-fibrous carbohydrate concentrations and fiber digestibility similar to legumes, and could be also considered a relevant alternative species, especially when NO_3^- leaching from the root zone is problematic. In this research, the perennial grass and SB-forb had greater soil organic N than forage legumes, enhancing soil C sequestration and quality.

When perennial forages systems are analyzed, we must think them as holistic systems where trade-offs between the use of soil, plant, and feed resources, animal

performance, and GHG emissions must be taken into account. There are numerous environmental benefits of maintaining perennial pastures ecosystems, such as enhancing soil C reserves. For instance, the greater daily CH_4 emissions seen in the MB system can be offset by greater soil C storage. Greater accumulation of soil organic C over time enhances resilience in the face of climate variability, long-term adaptation to changing climates, and increased production, biodiversity, and greater economic returns. Though the changes in soil C storage are slow, it was apparent from this research that initial accumulation of organic matter when cropped soils are converted to pasture systems is significant and measurable.

Producers, agronomists, researchers and public and private organizations need to be made more aware of the value of perennial forages in managing agricultural N. The urgent goal of this research was to minimize N losses under increasingly erratic and extreme weather due to climate change and reduce the environment impact of ruminant production systems while increasing the food production in the form of red meat produced per unit of time or land area, without costly chemical inputs. This research will inform life cycle assessments where the total GHG emissions (CH_4, N_2O and CO_2) is estimated for meat production.

There are implications of the present research for soil, plant, animal, and ultimately human health. Future investigations should focus on improving the agronomic traits of the legumes and forb used in this study. Selection should be carried out to improve the rates of establishment and regrowth, competitiveness with weeds, and long-

term persistence and production, comparable to alfalfa or grass pastures, thereby increasing acceptability and adoption by producers.

Another approach could be the breeding and selection of ruminants to more efficiently convert protein and energy to meat and milk, thereby reducing enteric CH_4 emissions without lowering cattle production. There is also a need for new information on the importance of ruminant diet selection, and management of excretion of C and N in manure, as well as manure utilization. Further study of plant secondary compounds, such as the effect of tannins structure and concentration is needed to manipulate and optimize their use and interactions with rumen and soil microbes, to reduce nutrient losses from animal systems. Tannins can enhance ruminant nutrition and the retention of dietary N in meat or milk, while minimizing enteric CH_4 emissions, without reducing fiber digestion or ruminant performance. Our overall goal is to improve the efficiency of N and C retention within forage-ruminant systems lessening the negative environmental impacts of ruminant production systems.

Lastly, the alternative beef production systems studied have the potential to increase economic and environment sustainability of ruminant production while maintaining or improving food production quantity and quality and sustaining society and the viability of rural communities. Marginal agricultural land that cannot be used for annual grain production can be an invaluable and profitable source of dietary protein via milk and meat production without contributing the environmental degradation of water and air. In particular, greenhouse gas emissions associated with beef production can be

greatly reduced by moving cow-calf production from low-quality rangeland or even

good-quality grassland or grass pastures, onto legume-based humid or irrigated pastures.

CURRICULUM VITAE

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- Instituto Nacional de Tecnología Agropecuaria (INTA EEA Bordenave), Argentina. September 2001-Current.

    ➢ Researcher and Extension Agent: Forages and pastures management and production, and beef cattle production.

    ➢ Research Coordinator at INTA Bordenave Experimental Station (Jan. 2012-Apr. 2014).

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    ➢ Chief of the Forage and Feed analytical laboratory at INTA Bordenave Experimental Station (Nov. 2006-Dec. 2011).

    ➢ Participation in regional and national projects of INTA Argentina in the area of Forages and Pastures: Introduction, evaluation and management of alternative forage species and cultivars.

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Technical Skills:

- Native Language: Spanish.

- Second Language: English (fluent).
- Effectively present technical information to wide variety of audiences (academic, farmers, general public).
- On-farm seminars.
- Data analysis and management.
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- Conduct of field agricultural research.
- Lab. forages analysis and interpretation of feed samples.

Education:

- PhD, Utah State University, Logan (Utah) August 2014 – April 2020;

  Major emphasis: Plant Science; Major Professor: Dr. Jennifer MacAdam.

  Project: Enhance the production and sustainability of pasture fed-beef using non-traditional legume forages:

   - Investigate effects on enteric methane emissions and soil quality in beef cattle pasture systems, comparing a perennial tannin-containing and a non-tannin legume with a grass.

   - Determine *in vitro* fermentation kinetics and actual dry mater digestion of tannin-containing and non-tannin legumes, a grass and a forb and their isolated fiber to understand the influence of fiber and secondary compounds on hays fed to cattle.

   - Compare nitrogen balances under controlled conditions over simulated long-term grazed pastures, including legumes with biological N_2-fixation as well as a non-fixing forb and grass species under a controlled environment, to evaluate the impact of N_2 fixation and plant secondary compounds such as tannins on nitrogen cycling.

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  Project: Influence of soil water status on arbuscular mycorrhizas in three perennial grasses in Central Argentina.

- BS: Universidad Nacional del Sur, Feb. 1994 – Aug. 1999;

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Publications:

1- Bolletta A., Villalba J.J., Dai X., and MacAdam J.W. 2018. *In vitro* digestibility and fermentation kinetics of six irrigated forage hays in the US Intermountain West. J. Anim. Sci Vol 96, E-Suppl. 3. pp. 218-219, https://doi.org/10.1093/jas/sky404.475.

2- MacAdam J.W., Beauchemin K., Bolletta A.I. and Pitcher L. 2016. Reduced Enteric Methane Emissions on Legume vs. Grass Irrigated Pastures. J. Anim. Sci Vol. 94, E-Suppl. 5/J. Dairy Sci. Vol. 99, E-Suppl. 1. pp. 315-316. doi:10.2527/jam2016_0660.

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5- Busso C.A., and Bolletta A.I. 2008. Soil phosphorus concentration under the canopy of perennial grasses of different successional stages exposed to water stress. Commun. Soil Sci. Plan. 39:2768-2783.

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