

Effects of Oscillating Crude Protein Content of Dairy Cow Diets.

THESIS

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Abstract

Overfeeding crude protein (CP) is a common practice in the dairy industry because it reduces the risk of a loss in milk; however, overfeeding CP can increase feed costs and negatively impacts the environment. We hypothesized that oscillating dietary CP concentrations over 2-d periods to equal the average concentration of a diet limiting in metabolizable protein (MP) for lactating dairy cows would improve milk protein yield and nitrogen use efficiency (NUE) over a diet limited in MP because oscillation should stimulate nitrogen (N) recycling to the rumen. Twenty-one Holstein dairy cows averaging 117 DIM were assigned to a treatment sequence in seven 3x3 Latin Squares with 28-d periods. The Positive Control contained 16.4% CP (MP allowable milk = 45.4 kg/d based on treatment mean DMI), the Negative Control contained 13.4% CP (MP allowable milk = 28.6 kg/d), and the Oscillating treatment consisted of a diet with 10.3% CP fed for 2 d followed by a diet with 16.4% CP fed for 2 d repeated over the 28-d period to average 13.4% CP. To determine how long (or if) cows would respond to the lowest CP diet (10.3% CP), 8 additional Holstein cows were fed the 10.3% CP diet for 5 d. Milk yield for cows fed the 10.3% CP diet decreased compared to cows fed the Positive Control beginning on the second day the 10.3% CP diet was fed, indicating that the diet was deficient in MP. Milk yield was similar for cows fed the Negative Control compared to cows fed the Positive Control. Because milk yield was similar for cows fed the Positive Control and Negative Control, the 13.4% CP treatments (Negative Control and

Oscillating treatment) may have met the MP requirements of the cows; therefore, interpretation of whether or not oscillation would improve milk yield, milk protein yield on NUE over a diet limited in protein is restricted. Milk urea nitrogen (MUN), NUE, urinary N (UN; % of N intake), fecal N (% of N intake) were similar for cows fed the Oscillating treatment and cows fed the Negative Control. There was a trend for a decrease in energy corrected milk (ECM) and milk protein and milk fat yields for cows fed the Oscillating treatment compared to cows fed the Negative Control. Milk yield decreased from the first day to second day cows on the Oscillating treatment were fed the 10.3% CP diet, explaining the trend for a reduction in milk protein yield and indicating that 2 d may have been too long for the diets to be fed before being switched. Oscillation at most maintained milk yield, milk protein yield, and NUE compared to the Negative Control.

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Chapter 1: Literature Review

Introduction

The nitrogen (N) cycle is required for life on Earth. The growing human population, as well as technologies, has changed the N cycle. Globally, recycling of manure N back to agricultural systems (excludes manure not recycled back to agriculture such as manure stored in lagoons) is estimated to have increased from 51 teragrams (Tg) per year in 1950 to 92 Tg per year in 2000 (Bouwman et al., 2011). With more people to feed, the need for more animal production arises. Dairy cattle and other production animals are a major source of N excretion into the environment. On average, a dairy cow producing 9,500 kg of milk/lactation excretes about 130 kg of N in 24 tons of manure annually (Weiss et al., 2009a). Typically, only about 30% of manure N is recovered per year and used as fertilizer for crops (Pinder et al., 2003). Further, 25% of dairy manure N can be volatilized as ammonia; the rest of the manure N can be denitrified or leach and run off into water sources (Pinder et al., 2003). Although animal excreta are a natural fertilizer, excess N from excreta causes pollution of water and air.

Nitrogen use efficiency (NUE) calculated as N secreted in milk divided by N intake is affected by many factors within the rumen such N intake relative to requirement. Some countries, such as the Netherlands, have put limits on N excretion and N fertilization because of public concern for the environment (Børsting et al., 2003). Therefore, nutritionists and other scientists have been researching different ways to reduce gaseous N emissions from production animal systems and increase NUE, including finding easier

ways to measure ammonia emissions and other outputs. However, maximum environmental and economic efficiencies for N use do not correspond (St-Pierre and Thraen, 1999). Nutrition, management, and genetic strategies to reduce N loss should maximize producer profits while considering the effect of N waste excreted into the environment.

Nitrogen in the environment

Importance of and changes to the nitrogen cycle

N is required for nucleic acid and protein synthesis. Although N is the fifth most abundant element on the planet, nitrogen gas (N_2) is inert. About 78% of the atmosphere is made up of N_2 ; however, the strong triple bond is hard to break (Galloway et al., 2004). As discovered in 1888 by Hellriegel and Wilfarth, some prokaryotes fix N from the atmosphere so it can be utilized by plants (Hu and Ribbe, 2011). The N cycle is made up of redox reactions, allowing N to exist in its various oxidation states (Figure 1). To incorporate N into biological molecules, N gas undergoes the process of fixation when it is reduced to ammonium. The enzyme nitrogenase catalyzes the reaction that converts N_2 to ammonia, a reaction that requires ATP (Burris, 1942). Many prokaryotes cannot fix N; therefore, they must obtain their N directly as ammonium or by reducing nitrates to ammonium. Ammonium is returned to the soil when organisms die and through excretions from animals. In the presence of oxygen, some species of Bacteria and Archaea oxidize ammonium to nitrates in a two-step process called nitrification (Galloway et al., 2004). The organisms that catalyze nitrification fall into two categories: ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Nitrification

was thought to mainly be a chemoautotrophic, obligatory aerobic practice that can only be done by a few groups of Proteobacteria (Francis et al., 2007). Recently, discoveries have indicated that ammonia oxidation can occur in anaerobic conditions, and aerobic ammonia oxidation can be accomplished by some Archaea. Without oxygen, nitrates can be reduced to ammonium in dissimilatory nitrate reduction or back to N_2 in the process called denitrification (Canfield et al., 2010). Denitrification is a heterotrophic, facultative practice accomplished by a wide range of genera (Francis et al., 2007). N_2 can also arise from the process called anaerobic ammonia oxidation (anammox), where ammonium oxidation is coupled with nitrite reduction by a certain genus of bacteria (Canfield et al., 2010). The ammonium in prokaryotes flows through the food chain, and is deposited back into the environment with animal excretions.

Fixed N, also known as reactive N, arises from agricultural actions, utilization of fossil fuels, and more recently from the use of biofuels. The production of reactive N grows every year due to the increasing human population of the Earth (Galloway et al., 2008). The creation of reactive N has increased from an estimated 15 Tg N/year in 1860 to approximately 186 Tg N/year in 2005 (Galloway et al., 2008). With the growing population of the Earth, demand for fixed N has increased due to the need to produce more food (Canfield et al., 2010). The rise in reactive N creation since the late 1800s corresponds to the increase in cereal and meat production in that same period. The large increase in reactive N production since the late 1800s was made possible by the development of synthetic fertilizers, and combustion of fossil fuels in industrialization also contributed to the rise in reactive N (Martínez-Espínosa et al., 2011). As of 2010, the

human contribution of fixed N, including that from animal production, is double the rate of natural N fixation and composes 45% of the reactive N produced on Earth on a yearly basis (Canfield et al., 2010). The growth of reactive N creation is not the only part of the N cycle that humans will change.

With the increase in fixation of N, microbes will eventually evolve to remove the excess N (Canfield et al., 2010). However, because the human population will continue to grow as well, a balance may take a long time to occur (Canfield et al., 2010). With the increase in fixed N, more N from river systems will travel to coastal regions and more ammonia will escape to the air, leading to environmental changes. Human additions to the N cycle have already caused an increase in the greenhouse gas nitrous oxide, as well as nitrates in soil, groundwater, and crops (Canfield et al., 2010). Natural biological N fixation (BNF) has been reduced due to the expansion of the Earth's population. BNF is estimated to contribute about 107 Tg N per year but is predicted to only contribute approximately 98 Tg N per year in 2050 due to changes in land usage (Galloway et al., 2004). Overall, human reactive N production was approximately 156 Tg N per year in the 1990s but is estimated to reach 270 Tg N per year by 2050 (Galloway et al., 2004). Human interaction, including the use of fertilizers and increasing numbers of production animals, has changed the N cycle, and these changes need to be taken into consideration for the future.

Environmental impacts of excess N

Although cattle manure can be used as plant fertilizer, N losses from cattle feces and urine can create problems, including run-off into water and ammonia volatilization in the

air. Urinary urea is a major environmental concern as urea in manure is hydrolyzed by urease to ammonia and can be quickly volatilized to the air, contributing to air pollution. Sixty to seventy-five percent of total urinary N is estimated to be urea N (Bristow et al., 1992). For dairy cows, on average about one quarter of manure N is lost as ammonia (Pinder et al., 2004). Ammonia contributes to eutrophication of bodies of water, acidification of the land, and human health problems (Burgos et al., 2007). Aquatic plants and algae require N, and they obtain N either naturally from minerals, lightning, or decomposition or from man-made sources such as humans and animal waste water, fertilizer, deposition of fossil fuels from the air, and polluted groundwater (Paerl, 1997). With excess N, phytoplankton blooms can be toxic to and reduce oxygen supply for other species, causing changes in the food web and killing fish (Paerl, 1997; Wolfe and Patz, 2002). Nitrates can leach into groundwater from soil, and nitrate pollution of groundwater in urban and rural areas can be caused by putting animal waste on soil (Prakasa Rao and Puttanna, 2000). After manure is applied as fertilizer, typically 20-40% of N loss from manure is in the form of ammonia, 1-25% is in the form of nitrates, and 1-4% is emitted as nitrous oxide (Rotz, 2004; Powell et al., 2010). Fertilizers can also directly increase nitrites and nitrates in crops (Prakasa Rao and Puttanna, 2000). For vegetables, the maximum admissible level of nitrite is 1 mg/kg of vegetables (Prakasa Rao and Puttanna, 2000). In developing countries, nitrate concentrations in food can be much higher (i.e. 20-76 mg/kg of cereals in India). Nitrates and nitrites in food can harm humans' health, especially infants (Prakasa Rao and Puttanna, 2000). Extra nitrites can lead to methemoglobinemia when nitrites reduce the oxygen-carrying capacity of the

blood. Methemoglobinemia is one of the reasons why nitrate levels in drinking water are not to exceed 45 ppm (Prakasa Rao and Puttanna, 2000; Wolfe and Patz, 2002). Manure from intensive animal production is one of the major contributors of nitrate leaching into groundwater, especially shallow groundwater (Infascelli et al., 2009). Nitrate toxicity from nitrate pollution of groundwater is also correlated with various forms of cancer, Alzheimer's disease, and neural tubes defects, among other health problems (Prakasa Rao and Puttanna, 2000). Nitrate and nitrite toxicity can cause methemoglobinemia in ruminant species as well (Prakasa Rao and Puttanna, 2000).

Nitrogen oxides are known to destroy ozone (Ravishankara et al., 2009; Wolfe and Patz, 2002). Nitrogen oxides are stable in the troposphere, but once they reach the stratosphere, they release chemicals which through nitrogen-oxide-catalyzed reaction destroy ozone. Although nitrogen oxides' ozone depleting potential is small compared to other ozone depleting substances, large anthropogenic emissions of nitrogen oxides make them harmful ozone depleting substances (Ravishankara et al., 2009). Anthropogenic emissions of nitrogen oxides are estimated to now be 10 million metric tons per year compared to about one million metric tons per year of chlorofluorocarbons (Ravishankara et al., 2009). Animal waste supplies 30-50% of global agricultural nitrogen oxide emissions (Oenema et al., 2005). Reducing the excretion of N from production animals is one way to limit the effects humans have on the N cycle. Improving NUE, while maintaining high production, is an economic and environmental concern.

Nitrogen Metabolism in Cattle

Basics on protein requirement

The aim of protein nutrition for dairy cattle is to provide enough rumen-degradable protein (RDP) to allow the rumen to function effectively and enough rumen undegradable protein (RUP) to provide amino acids (AA) to complement the microbial AA profile to optimize milk production and maximize long-term profit without overfeeding crude protein (CP). Historically, protein in a dairy ration has been represented by CP (NRC, 2001). Crude protein content of feed is calculated by multiplying the N content of the feedstuffs by 6.25. Amino acids, building blocks of protein, are utilized for maintenance, growth, and lactation of dairy cattle. The amino acids absorbed by the intestine are called metabolizable protein (MP). Metabolizable protein includes the microbial CP (MCP) synthesized in the rumen, RUP, and endogenous CP if these are digested (NRC, 2001). Previously, diets were formulated for CP, but using CP leads to inaccurate estimates of protein requirements because not all of the protein that is degraded by rumen microbes is synthesized into MCP and not all of the RUP and MCP that leaves the rumen are digested. Therefore, dairy rations are usually now formulated based on MP requirements.

Microbial crude protein synthesis

Feed is ingested and travels to the rumen where some of the protein is broken down. Microbes attach to the feed particles or the soluble N adheres to the bacteria, and microbial proteases break the protein into peptides and AA (Prins et al., 1979; Nugent and Mangan, 1981). About 20% to 40% of the total bacterial mass in the rumen is proteolytic (Prins et al., 1983). The wide array of bacteria in the rumen work together

using different proteases to catabolize feed protein (Wallace, 1985). *Ruminobacter amylophilus*, *Butyrivibrio fibrisolvens*, *Prevotella sp.*, and *Streptococcus bovis* are considered the primary proteolytic ruminal organisms (Wallace, 1985). The peptides and amino acids are transported inside the organism, and the peptides can be degraded to amino acids by peptidases (Tamminga, 1979). *Prevotella sp.* are the main organisms that break down peptides in the rumen (Wallace and McKain, 1991). These bacteria contain intracellular dipeptidyl peptidase (DPP). The DPP works by cleaving a dipeptide from the N-end of the oligopeptide. Dipeptides are cleaved off until only dipeptides and AA remain (Wallace and McKain, 1991). *S. bovis* and *R. amylophilus* are the two minor species that break down oligopeptides in another manner. They contain amino peptidases that cleave off single AA from the N-terminal end of the oligopeptide (Wallace and McKain, 1991).

Dipeptides and tripeptides are cleaved to AA by many bacterial species and some protozoa using dipeptidases. *Prevotella sp.*, *Megasphaera eldenii*, *F. succinogenes*, and *Lachnospira multiparas* are the main microbial species in this step of ruminal protein degradation (Wallace et al., 1996). The AA can be directly incorporated into MCP or can be deaminated to form volatile fatty acids (VFA), carbon dioxide, and ammonia. Both the dietary protein and nonprotein nitrogen (NPN) such as urea can be hydrolyzed to ammonia by ruminal microbes. Microbial species from previous steps of protein degradation such as *Prevotella sp.* play a role in deaminating AA. Some microbes have a preference for certain AA, but others have been shown to break down all AA (Scheifinger et al., 1976). However, the bacterial species from previous steps of protein

degradation are not the ones responsible for the majority of ammonia production in the rumen (Chen and Russell, 1988). A small set of gram-positive bacteria called ammonia-hyperproducing bacteria (HAP) have deaminase activity that contributes greatly to ammonia production in the rumen. The HAP include *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, and *C. aminophilum* (Attwood et al., 1998). Then rumen microbes use the ammonia or AA to synthesize MCP. Once inside the cell, the ammonia is assimilated into AA mainly by NAD-linked glutamate dehydrogenase (NAD-GDH) (Atasoglu et al., 1999). When ruminants are fed adequate N, achieving rumen ammonia concentrations from 5-15 mmol/L, NAD-GDH is the major mechanism for ammonia assimilation (Atasoglu et al., 1999). When rumen ammonia concentrations are ≤ 5 mmol/L, NADP-linked glutamate dehydrogenase and glutamine synthetase-glutamate synthase are the coupled reactions for ammonia assimilation (Erflle et al., 1977). Once the N is assimilated into glutamate, aminotransferases distribute the N to other AA (Ruhul Amin et al., 2002). The AA are linked together with peptide bonds to form MCP.

Protein intestinal digestion and absorption

The MCP and RUP flow to the abomasum and small intestine where some is digested. The average digestibility of MCP is 80%, and the digestibility of RUP ranges from 50-100%, depending on the feed (NRC, 2001). The digested protein, in the form of AA and small peptides, is absorbed across the wall of the small intestine and enters the blood stream (Owens and Bergen, 1983). The AA and small peptides are transported through the body and eventually enter cells to be synthesized into structural and

functional proteins, enter the mammary gland to be synthesized to milk protein, or are catabolized and the N is excreted from the body.

Urea recycling

Urea recycling and N in diet

Amino-N from AA not used by the cow can be converted to urea and then excreted, whereas the carbon skeleton is oxidized to carbon dioxide or used to synthesize other compounds. Excess ammonia in the rumen is absorbed across the ruminal wall and is transported in the blood to the liver, where it is converted into urea because excess ammonia in the blood is toxic (Owens and Bergen, 1983). The urea can either be excreted or recycled back to the rumen via saliva or active transport using urea transporters for microbes to use (Stewart et al., 2005). Urea is broken down into ammonia and carbon dioxide by the enzyme urease (Cheng and Wallace, 1979). Bacteria in the rumen digesta hydrolyze urea entering with saliva. Other species, specifically *Staphylococcus sp.*, live on the rumen wall and hydrolyze the urea that diffuses from the blood (Wallace, 1979). The ammonia from hydrolyzed urea can be used to make microbial protein (Kennedy and Milligan, 1980). Therefore, urea hydrolysis in the rumen allows for a higher % of N intake to be retained when ruminants are fed low N diets (Leng and Nolan, 1984).

More recycled urea is utilized by bacteria when diets are low in protein because efficiency of urea recycling, defined as the amount of captured recycled N over total N recycled, increases with lower dietary N intakes (Owens and Bergen, 1983; VanSoest, 1994). Ruminants become more efficient in using recycled urea because of physiological changes accompanying low dietary N concentrations. More urea is reabsorbed from the

kidney, and more urea is delivered to the GI tract when N levels are low in rations, promoting more urea recycling (Leng et al., 1984; Isozaki et al., 1994; Ford and Milligan, 1970). Transport of more urea to the GI tract may be due to the increase in urea transporter activity in the rumen lining (Ritzhaupt et al., 1997). At higher N intakes, the recycled N is diluted by the ammonia already degraded from feed protein, and recycled N is used less efficiently by the microbes (Castillo et al., 2001). A higher rumen ammonia concentration is usually associated with a low amount of capture of recycled N (Owens and Bergen, 1983; Abdoun et al., 2007).

Measuring urea recycling

One way understanding of urea transport and kinetics was gained was from studies using infusions of labeled urea. These studies examined urea entry rate into the rumen and urea output into urine with the difference being urea entering the digestive tract (Harmeyer and Martens, 1980). Nolan and Leng (1972) used [^{15}N]-labeled urea to estimate the rate of ureagenesis and amount of urea transferred to the whole digestive tract and to the rumen in sheep. Bunting et al. (1987) used both [^{14}C]-labeled urea and [^{15}N]-labeled urea to more accurately estimate the urea that was recycled. Another technique using intravascular infusions of [^{15}N ^{15}N]-labeled urea estimates urea recycling by analyzing three N isotopic species: [^{15}N ^{15}N], [^{14}N ^{15}N], and [^{14}N ^{14}N] (Lobley et al., 2000). The amount of [^{15}N] in the feces and [^{14}N ^{15}N] urea in the urine subtracted from the amount of total urea entry is an estimate of the urea that was reused.

Other studies have used veno-arterial measurements to estimate urea recycling (Lobley et al., 2000). Although surgery is involved, this technique can be used in studies

in which urea production may or may not be changing and can be used to look more closely at the metabolism of urea in a specific area like the rumen if catheters are placed correctly (Rémond et al., 1993). Other nitrogenous compounds can be measured at the same time with this technique as well, allowing researchers to determine the correlation between urea and other nitrogenous compounds for an indirect measurement of urea recycling.

Excess nitrogen

Efficiency of N utilization

The efficiency of feed utilization by dairy cattle can be defined many ways. For feed efficiency, the feed conversion ratio (FCR) and gross feed efficiency (GFE) can be measured (Zamani, 2012). FCR is defined as the ratio of dry matter (DM) intake divided by milk yield, and GFE is calculated as the ratio of milk yield over feed intake. Feed efficiencies for specific nutrients can be calculated. For CP (i.e. N), there is NUE or milk N efficiency (MNE). NUE is calculated by dividing the amount of N secreted into the milk by the amount of N consumed (Powell et al., 2010; Hristov et al., 2004). NUE can be defined in different ways. Manure NUE is defined as the amount of N taken up by crops divided by the amount of N applied via manure. Whole-farm NUE is the proportion of N exported off of the farm each year (includes milk, animals, manure, and crops) over the amount of N imported onto the farm each year (includes animals, fertilizer, purchased feeds and N fixed by legumes) (Powell et al., 2010). Efficiency affects profitability of the farm. A common way to calculate profitability is income over feed cost (IOFC), which is defined as the income from milk, milk protein, and milk fat yields minus the feed costs

(Zamani, 2012). For dairy farms, feeding to maximize IOFC is likely the most economically efficient approach (St-Pierre and Thraen, 1999). Lower producing cows excrete more manure N per kg of milk even though they excrete less N overall (St-Pierre and Thraen, 1999). A larger number of lower producing cows would be needed to produce the same amount of milk as fewer high producing cows, resulting in an overall increase in N excretion. Therefore, raising herd productivity should not only increase profit, but it usually increases NUE. Minimizing N excretion would benefit the environment but at the cost of milk production and profit (St-Pierre and Thraen, 1999). St-Pierre and Thraen (1999) calculated that it would cost \$4.40 per kg of reduction in N excretion (based on 1999 prices) because of loss in milk production. Thus, it is best for producers to feed cows to maximize productivity and profit. In efficient versus less efficient cows, more feed N will be transferred to milk N, including milk protein.

Factors that affect rumen ammonia levels and N efficiency in the rumen

Protein

Because about 40%-80% of the N used by ruminal bacteria comes from ammonia N, rumen ammonia concentration is the major factor that affects N utilization and microbial protein synthesis in the rumen (Mathison and Milligan, 1971; Hristov and Broderick, 1996). Only about 20-30% of dietary N is secreted as milk protein, and almost 50% of dietary N may be lost in the urine (Tamminga, 1992). About one-third of the N lost in urine is estimated to be due to inefficient N metabolism in the rumen such as inability of microbes to capture rumen ammonia (Tamminga, 1992). The concentration of ruminal ammonia, and therefore NUE, is a function of the degradability of CP and the

concentration of RDP in the diet. A low protein diet will usually result in a lower rumen ammonia concentration than a high protein diet. When dairy cows were fed a diet that met 80% of their RDP needs or a diet that met 110% of their RDP requirement, the cows on the high diet had a greater ruminal ammonia concentration than those on the low protein diet (Belanche et al., 2012). Cows on a diet with a higher CP concentration also have greater rumen ammonia concentrations than cows on a diet with lower CP concentration (Colmenero and Broderick, 2006; Hristov et al., 2004). Raising CP concentrations when diets are at requirement will typically result in a decrease in NUE as well. As CP increased from 16% to 17.5%, milk production over the whole lactation was not affected by dietary CP concentration, but NUE decreased from 28.8% to 24.7% (Wu and Satter, 2000). As CP content increased from 16% to 19% of DM (6% or 9% RUP) in diets fed to high-yielding dairy cows from -14 to 114 d postpartum, milk yield was on average equal for both treatments (39.8 kg/d vs. 42.4 kg/d, $p=0.33$), and concentration of milk protein increased from 2.89 to 3.08% as CP increased (Komaragiri and Erdman, 1997). NUE stayed at approximately 30% (30% versus 29% for low and high CP diets respectively) as CP% increased, but the similar NUE across dietary CP concentrations is an exception as NUE usually decreases as dietary CP increases (Komaragiri and Erdman, 1997). Increasing CP% of the diet will increase rumen ammonia levels, and not all of the ammonia will be captured by microbes to make MCP; therefore, the excess ammonia will be converted to urea in the liver. Much of that urea will be excreted into the urine, decreasing NUE.

Protein degradability has an effect on rumen ammonia concentrations also. A higher amount of rumen degradable protein will increase rumen ammonia concentrations compared to a lower amount of degradable protein and will usually decrease NUE if diets are at or above requirements for RDP. Increasing RDP from 9.5% to 11.9% increased rumen ammonia levels and decreased NUE from 30 to 25% (Armentano et al., 1993). Comparing levels of RDP on NUE, Calsamiglia et al. (1992) fed either a TMR formulated to contain RDP at 13.4 or 10.8% of dietary DM (at same concentration of CP 19.7%) to 48 multiparous cows from 31 to 180 DIM. Fishmeal and expeller processed soybean meal were used to replace soybean meal in the less degradable diet. Concentration of RDP did not affect milk production, plasma urea N, or NUE (Calsamiglia et al., 1992). Degradability of CP and CP% of the diets were unexpectedly similar to each other in this study, meaning that both diets exceeded the NRC requirements for the cows used in this study, which was most likely the cause for the similar NUE and milk production results between the two diets (Calsamiglia et al., 1992). When MP and RUP were held constant, cows on a high RDP diet of 11.6% showed a 25% increase in ruminal ammonia concentrations compared to cows on an adequate RDP diet of 9.4% and a decrease in NUE (Hristov et al., 2004). To determine the proportion of milk N from bacterial or ammonia N and the proportion of bacterial N from ammonia N, rumen ammonia was labeled by pulse-dosing with $(^{15}\text{NH}_4)_2\text{SO}_4$ (Hristov et al., 2004). The amount of ammonia N in milk protein as a proportion of total intraruminally-dosed ^{15}N was higher for the low RDP diet, and the cows fed the diet containing the greater amount of RDP had higher urinary N excretions (Hristov et al.,

2004). With high levels of RDP, most excess rumen ammonia is converted to urea and excreted; only a small amount will be used to synthesize nonessential amino acids and potentially be incorporated into milk protein; hence, NUE will decrease.

Increasing the concentration of RUP in dairy cow rations often decreases NUE. Increasing CP from 16.45 to 19.4% by changing the amount of RUP in the diet did not affect milk yield but did decrease NUE from 36% to about 31% (Christensen et al., 1993). Cunningham et al. (1996) replaced corn grain with solvent-extracted soybean meal to increase CP% from 16.5% and 18.5%. For the 16.5% and 18.5% CP diets, some of the solvent-extracted soybean meal was replaced with specially processed soybean meal to obtain a high and low concentration of RUP within each of the two CP levels (6.2, 7.3, 6.7, and 8.3% RUP). NUE decreased from 30% at 16.5% CP to 27% at 18.5% CP with milk yields similar at 37.1 kg/d versus 37.5 for the 16.5% and 18.5% CP diets respectively with no effect of RUP level (Cunningham et al., 1996). The rumen ammonia concentration increased as degradability and amount of dietary protein increased (Cunningham et al., 1996). Therefore, cows fed a diet with RDP over the requirement for ruminal microbes will utilize ruminal ammonia less efficiently than those fed the required amount of RDP.

Carbohydrates

The type of carbohydrate will also affect rumen ammonia concentrations and NUE. Cows on a high fiber diet had a 2.3-5.8 times higher rumen ammonia concentration than those cows on a high starch diet with CP concentrations equal between diets (Belanche et al., 2012; Calsamiglia et al., 2008). The ammonia peak at 2.5 h after feeding was also

greater for cows on the high fiber diet. Belanche et al. (2012) asserted that the microbes are better able to capture N when cows are fed highly degradable carbohydrates, resulting in a lower ammonia concentration in the rumen. Diets with higher amounts of starch or glucose in the forms of cornstarch and corn dextrose decreased the cows' ruminal ammonia concentration more so than diets with a large amount of fiber in the form of oat fiber (Hristov et al., 2005). Glucose may decrease the ammonia concentration by decreasing ammonia production in the rumen due to a low pH, causing a decline in the activity of fibrolytic bacteria. In contrast, starch may decrease the ammonia concentration by increasing the uptake of ammonia for MCP as indicated by the fact that the flow of microbial N from ^{15}N -labeled ammonia was greater for the starch diet compared to the glucose or fiber diets (Hristov et al., 2005). The main difference in microbial N synthesized from ammonia N was between the glucose and starch treatments. With glucose there was a faster release of ruminally fermentable energy compared with starch, causing a decreased degradation of alfalfa AA (Hristov et al., 2005). The decrease in degradation of AA increased uptake of preformed AA rather than ammonia N with the glucose diet. Further, the least amount of uptake of ammonia N for MCP was seen with the NDF diet because both starch and glucose allow for faster availability of ruminally fermentable energy (Hristov et al., 2005).

In studies comparing different starch degradabilities by using dry-rolled versus steam-flaked corn or sorghum, a trend for increased microbial protein synthesis (MPS) with steam-flaked corn or sorghum was seen (Theurer et al., 1999). The increase is due to increased starch degradation with steam- flaked corn rather than dry-rolled corn (Theurer

et al., 1999). Comparing steam-rolled corn versus barley diets (concentrate 60% of diet DM), Yang et al. (1997) showed that even though rumen ammonia concentrations were similar, microbial N flow to the duodenum increased from 48.5% to 54.6% of NAN with the barley diet compared to the corn diet. The increase in MPS resulted from the increased amount of OM and starch digested in the rumen with the barley diet, leading to more energy available for MCP (Yang et al., 1997). When barley and corn were fed to dairy cows at 42% or 39% of dietary DM respectively, barley decreased rumen ammonia concentrations, but microbial N outflow from the rumen was equal for both grains (Casper et al., 1999). Using four crossbred Holstein–Friesian heifers in a 2 x2 cross-over design, starch degradability (barley versus corn) over different concentrations of RDP did not affect rumen ammonia concentrations; however, the more degradable starch in the form of barley did increase duodenal flow and efficiency of MPS (Martín-Orue et al., 2000). Higher rumen ammonia concentrations should arise from barley compared to corn diets because of the higher concentration and degradability of barley protein compared with corn protein (Herrera-Saldana et al., 1990). The starch from barley is also more degradable than starch from corn; therefore, there should be more energy available from barley for the ammonia to be utilized for MCP, resulting potentially in greater NUE for barley compared to corn diets.

However, the effect of starch source on ruminal ammonia levels is variable and many times has no relationship with NUE. For fiber sources, concentration of fermentable carbohydrates in forage crops varies diurnally with more water-soluble carbohydrates (WSC) in forages cut in the afternoon compared to the morning and less WSC available

as the season continues (Bowden et al., 1968). Lee et al. (2002) showed that the greater amount of fermentable substrate with high-total nonstructural carbohydrate forages can increase rumen ammonia utilization in steers (Lee et al., 2002). High-WSC ryegrass increased NUE compared to low-WSC ryegrass in lactating dairy cows (Miller et al., 2001). Therefore, degradability and type of carbohydrate will affect rumen ammonia concentrations and NUE.

Although a minor factor, some of the protein from plants is trapped within a fiber matrix, and the type of diet will change the activity and number of different microbes that can digest the matrix (Bach et al., 2005). For instance, if a concentrate diet is fed to an animal, the pH will decrease. The decrease in pH may not affect the proteolytic bacteria, but it would most likely reduce the amount of cellulolytic bacteria, which would not be able to degrade the fiber matrix as well (Bach et al., 2005). Therefore, not as much protein would be degraded, resulting in a lower ruminal ammonia concentration. Types and amounts of bacteria, carbohydrates, protein, along with ruminal pH, all combine to affect the ruminal environment and change the concentration of ammonia present in the rumen.

Interaction between protein and carbohydrates

The utilization of ruminal ammonia depends on both carbohydrate and N availability and the rate of release of ruminal ammonia. Microbes will degrade protein to ammonia but ammonia assimilation will be inhibited if energy is limiting (Nocek and Russell, 1988). The experiments testing the concept that maximal microbial protein synthesis will occur if degradation of carbohydrate and protein sources is synchronized

have varying results. Two diets were formulated to be either synchronous or asynchronous in supply of energy and protein for sheep; the synchronous diet increased estimated production of microbial N by 27%, and increased microbial protein efficiency (microbial protein over dietary protein) by 13% (Sinclair et al., 1993). Similar results were reported when a synchronous or asynchronous diet with similar carbohydrate compositions were fed to sheep with microbial protein efficiency increasing by about 10% for the synchronous diet compared to the asynchronous diet (Sinclair et al., 1995). Supplementing a grass silage and grain concentrate basal diet with intraruminally infused maltodextrin synchronously (started with meal) or asynchronously (started 6 hours after meal) did not affect NUE (Kim et al., 1999). When cattle were fed diets with a source of slowly degradable carbohydrates (fiber) or a source of rapidly degradable carbohydrates (starch) at either synchronous or asynchronous release of N, plasma ammonia levels were higher in cows fed diets with asynchronous release of N compared with synchronous release of N (Sinclair et al., 2000). In a similar study with growing lambs, synchrony did not affect ruminal ammonia levels, estimated microbial protein synthesis, nor NUE (Richardson et al., 2003). Both N and carbohydrates have to be available for MCP synthesis in the rumen. Ammonia production can exceed the rate of uptake by the microorganisms if there is a lack of fermentable energy (Sauvant and Milgen, 1995). Rumen ammonia concentrations can be decreased by increasing carbohydrate degradability or decreasing N degradability (Sinclair et al., 2000; Kim et al., 1999). Though both energy and N are required for MCP synthesis, synchronizing degradability of carbohydrates and protein sources has rarely affected MPS or NUE, and any increase

in MCP may be due to the different feed components themselves and not the synchronization. Overall, dietary factors that affect the ruminal ammonia concentration are the amount of RDP, the rate of ruminal protein degradation and the availability of energy for the microorganisms (Hristov et al., 2004).

Lipids

Dietary lipids can also affect N metabolism and efficiency by impacting protozoa, microbial fermentation and intake (Jenkins, 1993). When dairy cows were fed diets containing either no added fat or 4.5% added fat with varying fat sources, the diets with tallow fatty acids had almost 40% higher efficiency of MPS (Jenkins and Palmquist, 1984). Three diets were fed to wethers with either no added fat, 5.2% soybean lecithin, or 2.4% corn oil; the added fat diets decreased ruminal ammonia concentration but increased N flow to the duodenum and increased efficiency of microbial protein synthesis (Jenkins and Fotouhi, 1990). Some studies with dairy cows have resulted in no difference on NUE when fat is supplemented. For instance, feeding 0, 2.5% or 5% added fat in the form of animal-vegetable or calcium soaps to ruminally and duodenally cannulated dairy cows did not affect rumen ammonia concentration nor efficiency of microbial protein synthesis (Ohajuruka et al., 1991). A control hay-concentrate diet or the control diet supplemented with 5% or 10% rapeseed oil or 10% tallow was fed to four cows in a Latin Square design (Doreau et al., 1991). The added fat only reduced rumen ammonia concentrations at one of the four sampling times and had no effect on the efficiency of microbial protein synthesis. According to a review on maximizing microbial protein synthesis (Firkins, 1996) fat addition, especially using unsaturated fats, will usually

decrease protozoa numbers. Reducing protozoa numbers can decrease NUE because protozoa ferment bacterial proteins and decrease ruminal ammonia levels.

Nitrogen excretions

Milk Urea Nitrogen

Urea from ammonia that was not used in microbial protein synthesis is excreted. AA are deaminated and the N from AA catabolism is converted to urea, which is also excreted mainly in the urine (Gehman, 2011). Less than 1% of urea transported in the blood will be secreted into the milk (Bannink et al., 2013). Therefore, a high MUN concentration (above 15 mg/dL, although it varies by herd) means that feed N is not being converted into milk protein efficiently (Gehman, 2011). Similar to NUE, MUN is related to variability of energy and N, as well as protein degradability and rumen ammonia levels. Ropstad et al. (1989) fed dairy cows diets containing either high or low protein (12.5% RDP or 17.5% RDP) with high or low fat. By using an analysis of variance, the researchers found that protein balance in the rumen, intake of RDP, and rumen ammonia levels were significant sources of variation in MUN (Ropstad et al., 1989). Diets varying in degradable to undegradable protein ratios (% of RDP:%RUP relative to NRC requirements were 80:80, 100:100, 120: 80, 100:120,120:120) showed that as both degradable and undegradable protein increase above requirements, plasma urea nitrogen (PUN) concentration will increase (Roseler et al., 1993). Increases in either undegradable or degradable protein raise MUN levels to a similar extent. Milk NPN concentration rose as protein levels exceeded requirement mainly due to an increase in urea in milk (Roseler et al., 1993). More discussion of nutritional factors that affect MUN

will be in the section detailing how MUN can be used as a tool to assess the protein status of cows.

Non-nutritional factors that cause variation in MUN include genetics, time of sampling, and stage of lactation. A study including 177 dairy farms on Prince Edward Island indicated that MUN is low for the first month of lactation and decreases during late lactation after a peak at four months (Arunvipas et al., 2003). Across a lactation, MUN increases as milk yield increases and decreases as milk protein concentration increases (Arunvipas et al., 2003). Variation due to cow, herd, and month sampled also occurred with total non-nutritional variation of MUN estimated to be 13.3% of total variation in MUN (Arunvipas et al., 2003). Aguilar et al. (2012) wanted to determine the effect of cow and herd variation on MUN. They found that cow does have a significant impact on MUN, suggesting that there are phenotypic differences in MUN among cows (Aguilar et al., 2012). MUN is heritable according to Mitchell et al. (2005), and phenotype of MUN may be due to differences in genotypic differences among urea transporters, affecting the number and activity of urea transporters. Diurnal variation in MUN also exists as MUN is higher directly after feeding, peaking 1-2 h after feeding (Gustafsson and Palmquist, 1993). Diurnal variation of MUN corresponds to the diurnal response of blood urea nitrogen (BUN) as MUN equilibrates with BUN as urea diffuses into the mammary gland. Although nutritional factors are the main contributors to MUN variation, non-nutritional factors still need to be considered especially when using MUN to assess the protein status of a herd.

N in manure

Dairy cattle excrete double or triple the amount of N into the manure when compared to milk (Broderick, 2005). When dairy cows were fed three diets containing amounts of increasing CP (15.1%, 16.7% and 18.4% CP) within three levels on NDF, there was a linear rise in estimated urinary N excretion from 140-236 g N/day (Broderick, 2003). The increase in total urinary N excretion was accompanied by a 69% increase in urine urea N with urea N as the main form of N found in the urine (Broderick, 2003). Increasing CP by adding soybean meal did increase milk N by a small amount, but the larger change in N excretion with the increase in soybean meal was the decrease in fecal N output, directing the N to urinary urea excretion. Urinary urea and total N concentrations fell as NDF decreased in the diets, corresponding to the increase in NUE from 25 to 30% as NDF decreased from 36 to 28% DM. With the greater amounts of CP intake, NUE decreased from 30 to 24% (Broderick, 2003). Similar results for N excretion were seen when grazing Holstein-Friesian cows were supplemented with 1 or 6 kg of a high protein concentrate or 6 kg of a low protein concentrate (Mulligan et al., 2004). Cows fed the 6 kg of high protein concentrate had the highest N intake, total N, and urinary N excretions, and cows fed the 6 kg of the low protein concentrate had the lowest proportion of N excreted in the urine (54% versus 66% urine N/ total N excretion for 6 kg low versus 6 kg high protein concentrate). This and other studies have reported that as fermentable organic matter (OM) increases with decreasing CP, there is more incorporation of RDP into microbial protein, which means that there will be less urea from excess rumen ammonia excreted into the urine and potentially more N from MCP

excreted into the feces as not all of MCP is digested. The low protein supplemented cows received a diet higher in fermentable OM from sugar beet and citrus pulps than those cows fed the 6 kg high protein concentrate (Mulligan et al., 2004). The low CP concentration and high fermentable OM in the low protein concentrate allowed the cows fed the low protein concentrate to excrete less urinary N than cows fed the 6 kg high protein concentrate (Mulligan et al., 2004). Castillo et al. (2001a) reported that less rumen degradable sources of cereal starch (cornstarch) reduced urinary N excretion compared to more rumen degradable sources of starch (barley starch). The decrease in urinary N excretion could be due to the fact that urea is diverted to support microbial protein synthesis in the large intestine when starch is less ruminally degradable in the rumen, causing N excretion to be diverted to the feces rather than the urine (Castillo et al., 2001). When gradually increasing CP in 1.5% increments from 13.5% to 19.4% CP, MUN and urinary urea increased with the linear decline of NUE as CP increased (Colmenero and Broderick, 2006). Urinary urea N increased from 60 g/day to 210 g/day as CP increased from 13.5% to 19.4% CP, and because urine urea can be easily converted to ammonia, it is a major environmental concern.

Ways to reduce nitrogen waste

Nutrition

MUN

Because urea diffuses from blood to milk, milk urea is correlated with blood urea concentration and; therefore, the average amount of N that is absorbed but not used in the body. MUN can be used as a tool to determine the protein status (whether cows are being

fed protein at, above, or below requirements) of cows. One of thirteen diets varying in energy and protein were fed to 125 cows for 16 wk, and N losses were estimated using the DVE-OEB system (Hof et al., 1997). In the DVE-OEB system, DVE is the true protein digested in the small intestine, and OEB is the surplus N available for microbial growth. MUN ranged from 9.0 to 18.3 mg/dL with flow of CP out of the rumen (estimated by $N = OEB / 6.25$) as the main factor in variation of MUN bulk tank samples but not for individual cows (Hof et al., 1997). Cow variation was the main factor in MUN variation for individual samples. Therefore, bulk tank samples could be utilized to indicate the average amount of N loss in the rumen (Hof et al., 1997). Schepers and Meijer (1998) used the DVE/OEB system to evaluate protein utilization using MUN for 11 feed trials. They found a strong correlation between MUN and RDP balance in the diet. RDP balance was defined as the balance between the amount of microbial protein potentially synthesized from the amount of RDP in the diet and the amount of microbial protein potentially synthesized from the amount of energy available from fermentation. Although parity and stage of lactation did not significantly change MUN, there was a large variation in MUN among and within cows, making bulk tank samples - but not individual milk samples - a tool to assess the RDP balance in the diet (Schepers and Meijer, 1998). In order to develop a model that uses MUN to predict N excretion and NUE, Jonker et al. (1998) used three separate trials to develop the model and an additional 19 to evaluate the model. The model included milk production, MUN, CP in the diet, and milk protein concentration. The model was able to estimate N excretion and NUE with a 15% prediction error for the data sets used to evaluate the model. Even with

the model prediction error, most unexplained model error seemed to arise from cow to cow variation. Therefore, the researchers determined that using no less than 10 cows was adequate to estimate a target MUN concentration based on expected urinary N excretion for Holstein cows (Jonker et al., 1998). Fifty production trials from Finland and Sweden were used to evaluate MUN as a tool to evaluate cows' protein status (Nousiainen et al., 2004). Similar to Hof et al. (1997), dietary CP concentration was the greatest factor affecting MUN. Adding other factors along with CP intake did little to improve prediction of MUN. MUN was better able to predict UN excretion than NUE. The researchers proposed that MUN can be used to diagnose protein status of cows, as well as UN excretion. Due to high variation among and within cows, individual urea concentration from milk samples are not a good indicator of protein status in lactating dairy cows; however, an average or bulk tank MUN is a diagnostic tool for protein feeding and UN excretion, giving producers and nutritionists a non-invasive way to reduce excess N excretion.

Reduction of Dietary N

Reducing the amount of N fed to dairy cows is another nutritional way to reduce N emission. One way to reduce the amount of N fed to cattle is to use phase feeding, meaning that different amounts of protein are fed according to the cattle's stage in life (i.e. calves versus yearling for beef steers) instead of one protein level for all stages. Phase feeding feedlot cattle instead of feeding the standard 13.5% CP to growing cattle decreased N excretion by 13 to 22% without affecting weight gain (Klopfenstein and Erickson, 2002). By feeding cows closer to requirement, cows were able to increase milk

production when CP concentration decreased from 20% to 18% CP due to an increase in protein efficiency (Klausner et al., 1998). The reduction in CP also allowed for a drop in N excretion by almost 35% (Klausner et al., 1998). According to long-term trials, dairy cows producing up to 40 kg/d of milk are able to be fed balanced diets with a little as 16% CP without decreasing production; these 16% CP diets meet the MP requirements of the NRC with approximately a 10 g/d MP balance (Hristov and Giallongo, 2014). In long term 10-week studies, diets with 14% CP reduced production in cows producing 39 to 43 kg/day milk compared to cows fed diets with 15-16% CP due to decreased total tract NDF digestibility. However, supplementing 14% CP (MP-deficient) diets with rumen-protected amino acids maintained milk production (Lee et al., 2012b). Even though diets were around 5% deficient in MP according to the NRC, no reduction in milk production or DMI compared with the control was seen when the deficient diets were supplemented with slow-release urea, rumen protected methionine and histidine (Giallongo et al., 2014). Many short-term experiments with low protein diets (below 14% CP) do not show a significant reduction in DMI or milk yield; however, a decrease in DMI is a long-term effect of low protein diets (Hristov and Giallongo, 2014). Therefore, short-term Latin square studies may not always show a decrease in milk yield when dietary CP concentrations decrease because diets may not have been fed long enough to decrease DMI. For instance, when decreasing the CP% from 19.4 to 13.5%, milk yield and DMI were not statistically affected (Colmenero and Broderick, 2006). However, there was a trend for a decrease in DMI and milk yield for the 13.5% diet compared to the 16.5% CP diet. Therefore, loss in milk from low-protein diets results from depressed DMI from a

deficiency in RDP, which may decrease fiber digestion, and from not enough of the limiting AA for milk protein production. Hence, diets that are about 5% below the NRC's MP requirement or are around 13% below the requirement but are supplemented with rumen-protected amino acids can maximize milk production without overfeeding CP.

Oscillation

Another way to potentially increase NUE may be to oscillate crude protein concentrations in the diet. Although diets with oscillating crude protein have not been studied with dairy cattle, studies have been done with beef cattle and smaller ruminants. When lambs were fed a diet that oscillated between 10% and 15% CP on a 24-h basis, N retention was the same compared to the lambs on a continuous 12.5% CP diet (Cole, 1999). However, when the diet oscillated on a 48-hour basis, N retention was higher for animals on the oscillating diet. In one trial, oscillating CP to average 12.5% CP increased N retention by 38% when compared to the 12.5% continuous diet (Cole, 1999). Calculated as a percentage of N absorbed, N retention increased, indicating that more N was retained due to a higher utilization of absorbed N (Cole, 1999; Kiran and Mutsvangwa, 2009). The main mechanism thought to be the cause of the increased N utilization in oscillating diets is increased N recycling to the rumen. When steers were fed a diet that oscillated between 10% CP and 14% CP on a 48-hour basis, the steers on the oscillating and 12% continuous diet had lower urinary excretion of N compared to steers on the continuous 14% CP diet (Cole et al., 2003). The oscillating group also had greater protein retention compared to steers in the 12% continuous group. Overall, oscillating the

CP concentration in growing ruminant diets can improve N retention and reduce N excretion, potentially decreasing the effects of N emission to the environment and increasing NUE.

Management/Facilities and Genetics

A mathematical model created by Kohn et al. (1997) predicted that improving herd management is a likely way to reduce N loss to the environment. (Kohn et al., 1997). The model predicted an almost 15% increase in whole-farm N efficiency and a 15% decrease in N loss if loss from manure storage and application were reduced by 100%, indicating management strategies to reduce N loss from using manure as a fertilizer could increase total-farm N efficiency (Kohn et al., 1997). Crop selection and management could yield as much as a 60% increase in total farm N efficiency and decrease N losses from the animals by 40%. Appropriately grouping cows has been shown to reduce N excretion per cow by up to 8% per day (St-Pierre and Thraen, 1999). Although the end goal is the same, strategies that aimed to improve the accuracy of feeding N such as using forage analysis were actually less effectively used to decrease N loss compared to those strategies that aimed to improve production such as milking 3 times per day (Kebreab et al., 2002). In all, management strategies on the whole farm not just with lactating cows plus not overfeeding CP would greatly reduce the amount of N excreted into the environment.

With the increased awareness of the negative impacts of N loss from production animals on the environment and the variability in NUE evidenced among cows, interest in whether or not MUN or NUE can be selected for genetically has grown. Specifically

looking at MUN because it is a factor to predict the protein status of cows, heritabilities of MUN ranged from 0.45-0.60; however, there was little evidence for MUN to be correlated with milk yield or other economically important parameters (Wood et al., 2003). In another study, heritability of MUN was lower at 0.14, and again MUN was not highly correlated with milk production parameters such as milk yield (Stoop et al., 2007). Heritabilities of MUN ranged from 0.38-0.41 when the genetic correlation of lactose and MUN on production measures was studied (Miglior et al., 2007). Although MUN was not correlated with milk yield, it was positively correlated with fat and protein concentrations. In order to genetically evaluate the potential pollution of dairy cows to ground water resources, non-milk N was estimated, cows of average genetic merit for production had higher non-milk N per kg of milk than those of higher genetic merit for production (Chagunda et al., 2009). In pasture systems, Holstein-Friesian dairy cow strains selected for fertility and production (high-durability North American and New Zealand strains) had less N loss and higher profitability compared to the strain selected just for production (high-production North American strain). Genetics has the possibility of reducing N loss, but selecting for more efficient N utilizers may or may not be correspond to milk and milk component production traits.

Summary

The N cycle has been greatly impacted by human sources, including animal production, in recent decades. N that is not utilized by dairy cows and other production animals will be exerted in the manure, and excess ammonium, nitrates, and nitrous oxides, as well as other nitrogenous compounds, detrimentally impact the environment.

Increasing N efficiency in the rumen is one way to reduce N loss from animal production systems. N efficiency in the rumen is affected by the rate and amount of rumen ammonia produced. Therefore, degradability of protein and carbohydrate sources, the balance between protein and energy, the source of lipids, N recycling to the rumen among numerous other factors influence the rumen ammonia concentration, as well as NUE of the lactating dairy cow.

The distinction between environmental and economic N efficiency is important because reducing N loss to the environment as much as possible will likely reduce profits for dairy producers. There needs to be a balance between the N efficiency that is beneficial to the environment and to the producer. Nutritionists and other scientists have been developing ways to reduce N loss from ruminants on a whole-farm level. One way to potentially increase NUE is to oscillate crude protein concentrations in the diet. We hypothesized that oscillating the CP concentration to equal the average concentration of a diet limited in CP for lactating dairy cows will improve milk protein yield and NUE compared with a diet limiting in MP with constant MP because oscillating the CP concentration should increase recycling of N back to the rumen. Our objective in this study was to determine the effect of oscillating CP on milk yield, milk protein yield, and milk fat yield, as well as N excretion into milk and urine and NUE.

Chapter 2: Introduction

Overfeeding crude protein (CP) to dairy cows in the United States is common (Jonker et al., 2002) to reduce the risk of a loss in milk; however, overfeeding CP increases costs on the dairy (VandeHaar and St-Pierre, 2006; Colman et al., 2011) and negatively impacts the environment (Hristov et al., 2006). Based on a mail survey, dairy cows in the Chesapeake Bay Drainage Basin were fed on average 7% more CP than recommended by the NRC (Jonker et al., 2002). The average whole-farm nitrogen (N) surplus per cow for eight dairy farms in Idaho was calculated to be 169 kg/year (Hristov et al., 2006). On average, only 20-30% dietary protein is secreted in milk (Tamminga, 1992).

Recycling of N back to the rumen can increase the proportion of dietary N secreted in milk (Marini and Van Amburgh, 2003). Urea can be recycled back to the rumen either directly through transporters or through the saliva (Stewart et al., 2005). The recycled urea can be degraded and used to make microbial crude protein (MCP) by rumen bacteria and increase nitrogen use efficiency (NUE; N milk/N intake) (Kennedy and Milligan, 1980). Ruminant microbes become more efficient in capturing recycled urea when dietary concentrations of N are low compared to when dietary N concentrations are high because of the lower amount of N fed and physiological changes accompanying low dietary N concentrations (Leng et al., 1984; Isozaki et al., 1994; Ford and Milligan, 1970). Oscillating the crude protein content of diets may be a way to stimulate N recycling back to the rumen (Cole, 1999).

Feeding lambs an Oscillating treatment in which dietary CP concentrations switched between 10% and 15% CP every 2 d increased N retention by 38% compared to the constant 12.5% CP treatment in one trial but not another (Cole, 1999). The trial in which the Oscillating treatment had no effect used heavier lambs that had higher CP intakes than lambs in the trial that observed a positive response to the Oscillating treatment. Intake of rumen degradable protein (RDP) was estimated to be marginal for lambs in the trial that had a positive effect from the oscillation but was above requirements for lambs in the trial that found no effect. Ludden et al. (2002) reported that feeding wether lambs an Oscillating treatment (13% and 17% CP) did not increase N retention compared to those lambs fed a constant 15% CP diet. The lambs on the Oscillating treatment had higher total tract organ masses than those on the constant 15% CP diet (Ludden et al., 2002). The heavier organs would require more maintenance energy. Further, because infrequent (soybean meal fed every 3 d) protein supplementation can cause an increase in urea N output from the portal-drained viscera (Krehbiel et al., 1998), more energy is needed by the liver for ureagenesis. Therefore, the greater visceral organ mass of the lambs fed the Oscillating treatment could decrease energy efficiency, and therefore, protein efficiency (Ludden et al., 2002). In a separate study, steers fed an oscillating CP treatment (9% and 14% CP) retained more N than steers fed a constant 9% CP and 14% CP diets (Archibeque et al., 2007).

Studies evaluating the effects of oscillating dietary CP have not been conducted with dairy cows. We hypothesized that oscillating dietary CP concentrations to equal the average concentration of a diet limited in CP for lactating dairy cows will improve milk

protein yield and NUE because oscillating the CP concentration could increase recycling of N back to the rumen. The objective of the experiment was to determine the effect of oscillating dietary CP on milk yield, milk protein yield, and milk fat yield, as well as N excretion into milk and urine and NUE.

Chapter 3: Materials and Methods

Animals and Diets

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of The Ohio State University (Protocol # 2013A00000085). Twenty-one multiparous, mid-lactation, Holstein cows (117 ± 27 days in milk (DIM); 41 ± 7 kg/d milk yield) were used in the experiment. Cows were blocked by milk yield and by DIM, resulting in blocks with high or low producing cows in earlier or later in lactation. Each of the seven blocks was a 3x3 Latin square with 28-d periods. Cows were fed one of three diets after adjusting to the tie stalls for 6 d while being fed the Positive Control diet (Tables 1 and 2). Treatments were a Positive Control diet containing 16.4% CP (MP allowable milk (NRC, 2001) = 45 kg/d based on estimated 25 kg/d DMI), a Negative Control diet containing 13.4% CP (MP allowable milk = 29 kg/d), and an Oscillating treatment consisting of a low CP diet (10.3% CP; MP allowable milk = 17 kg/d) fed for 2 d followed by the Positive Control (16.4% CP) fed for 2 d repeated over the 28-d period (Figure 2) to average 13.4% CP (average MP allowable milk = 29 kg/day). Each period, 3 or 4 of the cows on the Oscillating treatment were first fed the 16.4% CP diet, and the other 3 or 4 were first fed the 10.3% CP. All diets contained 50% corn silage (Table 3), 10% alfalfa silage (Table 3), and 40% concentrate mix. Only 2 concentrate mixes were made (Table 4); one for the Positive Control and one for the 10.3% CP diet (the Negative Control concentrate was a 50/50 blend of those 2 mixes). To obtain different CP

concentrations, varying amounts of 2 soybean meals were replaced with a mix of soyhulls and corn grain. CP was replaced with a mix of 67% NDF and 33% starch. When soybean hulls replace corn grain as a source of energy in a diet, the concentration of nonstructural carbohydrates (NSC) in the diet decreases (Zervas et al., 1998; Ipharraguerre et al., 2002). However, studies have shown that readily digestible fiber from soyhulls are as effective as NSC from corn in providing fermentable energy for N digestion and synthesis of MCP as long as soyhulls are less than 30% of diet DM (Ipharraguerre et al., 2002; Ipharraguerre and Clark, 2003) . High levels of ruminally fermentable starch will increase volatile fatty acid (VFA) concentrations, lowering pH in the rumen and inhibiting microbial growth and fiber digestion, as well as potentially inhibiting MCP synthesis (Khorasani et al., 1994; McCarthy et al., 1989). About 80% of the nonfibrous carbohydrates in corn grain are starch (NRC, 2001); therefore, increasing the amount of corn grain in the diet by even a little would increase the starch concentration in the diet substantially. Hence, more NDF was used to replace protein than starch. RUP and RDP were manipulated to obtain the decrease in MP.

The Positive Control diet was formulated to meet all requirements (NRC, 2001) of the average cow one month prior to the start of the experiment (48 ± 5 kg of milk/day; fat = 3.7% and protein = 3.1%). The Oscillating-10.3 protein concentration was chosen because it was the lowest RDP and RUP concentrations possible with the feed components used in the Positive Control. The Oscillating-10.3 protein diet had an MP-allowable milk of 17 kg/d compared with 45 kg/d for the Positive Control. The Negative Control RDP and RUP concentrations were chosen to be the average between the

Positive Control and the 10.3% CP diet, resulting in a diet deficient (280 g/d below NRC requirement) in MP. The Negative Control had an MP-allowable milk of 29 kg/d (27% less than the average milk yield one month before the experiment). Cows were housed in tie stalls, and fed once daily with an average 8% feed refusal. Amount of feed offered and refused was measured daily to determine daily DMI. Cows were milked twice per day, and milk yields were recorded electronically. Cows were weighed on day 1 of period 1 and the last day (d 28) of each period.

Sample Collection and Analysis

Silage was sampled on Monday, Tuesday, Thursday, and Friday of each week with the Monday/Tuesday and Thursday/Friday samples composited into 2-d samples (Figure 3). A subsample of each 2-d composite silage sample was assayed for DM (100°C for 48 h) every Tuesday and Friday to determine whether or not diets needed to be adjusted. Subsamples of the 2-d composite samples were dried at 55°C and then ground using a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA) through a 1-mm screen, and then analyzed for CP (Kjeldahl (KJ) N x 6.25 (AOAC 984.13.4.2.09, 2000) every Thursday to determine whether or not the diets needed to be reformulated because they did not meet the CP specifications. No reformulation was needed because CP concentrations of diets did not deviate by more than 0.5 percentage units. As-fed amounts of the diet components were adjusted when DM of the either silage changed more than 5% units. A subsample of each daily silage sample was also frozen and composited into 2-wk composite samples. A subsample of all 2-wk silage samples was thawed and then dried and ground as described above. All dried and ground 2-wk silage samples were

assayed for DM (100°C for 24 h), ash (600° C overnight; AOAC, 2000), and NDF (Ankom200 Fiber Analyzer, Ankom Technology, Fairport, NY) with sodium sulfite and amylase (Ankom #FAA) weekly. The 2-wk composite corn silage samples were analyzed for starch (Weiss and Wyatt, 2000). The dried and ground 2-wk composite samples for silages were dry-ashed, and minerals were assayed using an inductively coupled plasma spectrograph (Service Testing and Research [STAR] Laboratory, Ohio Agricultural Research and Development Center, Wooster, OH). Corn and alfalfa silage particle size was measured on wet period composites using a Penn State Particle Separator (Lammers et al., 1996).

Samples of concentrates were taken every Monday and Thursday and were composited into 2-wk composite samples and frozen (Figure 3). When a new batch of concentrate was made, it was sampled and assayed for CP immediately (described above) to determine whether or not diets needed to be reformulated (diets were never reformulated due to CP content). The 2-wk composite concentrate samples were assayed for DM, ash, CP, starch and NDF as described above. Subsamples of the 2-week composite samples for concentrate samples were digested with perchloric acid and analyzed for minerals by STAR Laboratory as described above.

Period samples of the three total mixed rations (TMR) were made from the second dried, ground 2-wk composite samples of silages and concentrates of each period and were analyzed for 30-h in vitro NDF digestibility (Goering and Van Soest, 1970) by Cumberland Valley Analytical Services (CVAS; Hagerstown, MD).

Samples of feed refusals were taken for 4 consecutive days (one full oscillation cycle; see Figure 2) twice per period for all cows in the Oscillating treatment (d 6, 7, 8, 9, 22, 23, 24, 25 of each period) and for 2 d per period for all cows on the constant treatments (d 6 and 22 of each period). All samples were frozen until analyzed. All refusal samples were analyzed for DM in duplicate (100°C for 48 h). DM of refusals was used to calculate DMI. For DMI of the cows on the constant diets, DM of refusal was averaged by cow and the average DM was used for the whole period to calculate DMI. For cows on the Oscillating treatment, DM was averaged by cow for the days when the cows were fed the 16.4% CP diet and for the days when cows were fed the 10.3% CP diet. The diet average DM concentrations (within cow) were used to calculate DMI for each day the specific diet was fed. For each period, dried and ground refusal samples were composited by weight of refusal, resulting in a single composite sample for cows on the continuous diets and 2 composite samples for cows on the Oscillating treatments (one composite when the cows were fed the Positive Control diet and one composite when cows were fed the Oscillating-10.3 diet). All composite refusal samples for Oscillating cows were analyzed for CP (described above), and 4 refusal samples from 4 cows were randomly chosen for each of the two continuous diets per period to be analyzed for CP, resulting in 12 out of 21 samples analyzed for CP for each continuous treatment. The refusal CP data was used to estimate N intake.

Urine samples (~ 50 ml) were collected by vulva stimulation on d 21, 22, 23, 24 (1 full oscillation cycle per period; see Figure 2) for cows on the Oscillating and Negative Control treatments and d 21 and 22 for cows on the Positive Control treatment. Samples

were filtered through a paper towel and stored at -20°C until analysis. Thawed samples were warmed by heating at 37°C for 15 min in a water bath and homogenized by repeated pouring back and forth into a beaker. Samples were analyzed for creatinine (Cayman Chemical Item Number 500701, Ann Arbor, MI) and KJ N (AOAC 984.13.4.2.09, 2000). Urine excretion was estimated (Valadares et al., 1999) using creatinine as a urine marker: Urine excretion (L/d) = (29 mg daily creatinine excretion/kg BW)/ (analyzed creatinine in sample). Urine nitrogen excretion (UN) was then calculated as UN (g/d) = [Urine excretion (mL/d) x KJ N from sample (mg/mL)]/1000. Urine N was also estimated using MUN (Kauffman and St-Pierre, 2001): UN (g/d) = (0.0259 x kg BW x MUN (mg/dL)). The UN was then used to estimate urinary excretion: Urinary excretion (L/d) = [UN (mg/d)/KJ N of urine samples (mg/mL)]/1000.

Milk samples (A.M. and P.M. milkings) were collected on d 7, 14, 21, 25, 26, 27, 28 each period for determination of milk fat, milk protein, lactose, (B2000 Infrared Analyzer (Bentley Instruments, Chaska, MN) and MUN concentrations (Skalar SAN Plus segmented flow analyzer; Skalar Inc., Norcross, GA) by DHI Cooperative, Inc. (Columbus, OH). Milk yields from d 7, 14, 21, 25, 26, 27, and 28 were used to calculate yields of milk components. Milk samples were also collected for 3 full oscillation cycles per period for analysis of MUN (Figure 2). Because cows were fed after the A.M. milking, sampling started at the P.M. milking on the first day the oscillation cycle and ended with the A.M. milking on the last day of the oscillation cycle. Therefore, for the first full oscillation cycle: milk was sampled at the P.M. milking on d 3, the A.M. and P.M. milkings on d 4, 5, and 6, and the A.M. milking on d 7 (Figure 4). Milk sampling

during the other 2 full oscillation cycles (d 15,16,17,18, 19; 25, 26, 27, 28, and 29) followed the same sampling schedule as the first cycle sampled. Milk samples were refrigerated and then assayed for MUN within 24 h. Samples were warmed by heating at 37°C in a water bath and homogenized by repeated pouring back and forth into a beaker. Milk samples (3 mL) were then filtered to remove fat using a nylon, 0.45µm syringe filter (P.J. Cobert Associates, Inc. Item Number 9445511, St. Louis, MO) and analyzed for MUN (BUN kit STANBIO Laboratory, Enzymatic Urea Nitrogen Proc. No. 2050, Boerne, Texas adapted to 96-well plate reader). Prior to this experiment, this MUN assay was validated by taking milk samples from four cows. When whole milk was assayed for MUN, the wells became cloudy not allowing for accurate absorbance readings. Therefore, a subsample was centrifuged at 20,000xg for 20 min at 4°C, and the skim milk samples were assayed for MUN. Subsamples of whole milk were also filtered as above to compare MUN of filtered milk to MUN of skim milk samples. MUN from skim milk and filtered milk were comparable to MUN from DHI (i.e. skim: 16.7; filtered: 17.2; DHI: 15.9). Standards of urea nitrogen of 3.75, 5.25, and 7.5 mg/dL were added to the filtered samples to calculate recoveries. Recoveries were > 96%. In periods 2 and 3, composite samples from all cows were also composed from 10-ml subsamples of milk on days 25, 26, 27, 28, and 29 and frozen to be later analyzed for KJ N (AOAC 984.13.4.2.09, 2000) to compare the infrared method used by DHI for milk protein and the KJ method. KJ N was analyzed to compare NUE calculated from KJ N results to NUE calculated from DHI results. Fecal N was estimated by using the equation: Fecal N (g/d) = N intake (g/d) – (Milk N g/d + Urine N (g/d)).

Statistical Analysis

Data were analyzed with mixed models using the MIXED procedure of SAS (version 9.3, SAS Institute, Inc., Cary, NC). Block was not used in any of the models as block did not account for any of the covariance estimated when added to the model. Significance was declared at $P \leq 0.05$. Data is presented as LSMEANS.

To determine overall treatment effects for N intake, Milk N, NUE, UN and fecal N, all measurements were averaged within cow-period, and the averages were analyzed using the model: $Y = \mu + \text{cow (random effect, df = 20)} + \text{period (random effect, df = 2)} + \text{treatment (fixed effect, df = 2)} + \text{residual error (random effect, df = 38)}$. Single degree of freedom contrasts were used to compare the Negative Control to the Positive Control treatment and the Negative Control to the Oscillating treatment. The Positive Control vs. the Negative Control contrast was used to determine the effect of CP concentration (i.e. 16.4% CP vs. 13.4% CP). The Negative Control vs. Oscillating contrast was used to determine the effect of oscillation since on average the Oscillating treatment had the same CP concentration as the Negative Control.

The 2-d oscillations were chosen based on data from steers and sheep; therefore, the day effect was evaluated statistically to determine if 2-d oscillations were the appropriate length of time for oscillations in dairy cows. Milk composition was analyzed for 4 d per period (one full oscillation cycle). MUN was analyzed for 3 full oscillation cycles per period (12 d per period). Days for milk components, MUN, DMI and milk yield were recoded for all 28 d of each period (7 full oscillation cycles). Eleven of the cows fed the Oscillating treatment started on the Positive Control and 10 cows were first

fed the 10.3% CP diet; therefore, for cows that were first fed the 10.3% CP diet, d 3 and 4 of each cycle were switched to d 1 and 2. All cows on the Oscillating treatment would have d 1 and 2 for each oscillation cycle be days the cows were fed the Positive Control and d 3 and 4 days the cows were fed the 10.3% diet. To conclude that the actual date that cows on the Oscillating treatment were fed either diet did not matter, 3 cows on the Positive Control and 3 cows on the Negative Control were randomly chosen. Day 1 and 2 were switched with d 3 and 4 for the randomly chosen cows as well. To determine whether or not there was a treatment x day effect or treatment x day x cycle effect on DMI and milk yield a second model was used: $Y = \mu + \text{cow (random effect, df = 7)} + \text{period (random effect, df = 2)} + \text{treatment (fixed effect, df = 2)} + \text{day(cycle) (fixed effect, df = 18)} + \text{treatment x day (fixed effect, df = 6)} + \text{residual error}_1 \text{ (random effect, df = 38)} + \text{cycle (fixed effect, df = 6)} + \text{treatment x cycle (fixed effect, df = 12)} + \text{treatment x cycle x day (fixed effect, df = 36)} + \text{residual error}_2 \text{ (random effect, df = 1620)}$. Day was a repeated measure. MUN was analyzed using the same equation except for 3 cycles not 7 (cycle; fixed effect = 2). Milk protein, fat, and lactose concentration were analyzed using the equation for one cycle; therefore, cycle and its interactions were taken out the model. Single degree of freedom contrasts were used to compare the Negative Control to the Positive Control treatment and the Negative Control to the Oscillating treatment. The Positive Control vs. the Negative Control contrast was used to determine the effect of CP concentration (i.e. 16.4% CP vs. 13.4% CP). The Negative Control vs. Oscillating contrast was used to determine the effect of oscillation since on average the Oscillating treatment had the same CP concentration as the Negative Control. If a treatment x day

effect occurred ($P < 0.10$), then SLICE by treatment was used. For the Oscillating treatment only single degree of freedom contrasts were used to compare the first day Oscillating cows were fed the Positive Control to the second day Oscillating cows were fed the Positive Control (H1 vs. H2) and the first day Oscillating cows were fed the 10.3% CP diet to the second day Oscillating cows were fed the 10.3% CP diet (L1 vs L2).

For MUN analysis, milk samples were collected for a full oscillation cycle (a total of 8 milkings). Half of the cows on the Oscillating treatment were first fed the Positive Control diet and half were first fed the 10.3% CP diet. To compare the Oscillating treatment to the Positive Control treatment at any of the 8 milkings, the cows on the Oscillating treatment needed to be standardized. Therefore, the milkings were standardized so that all Oscillating cows were fed the Positive Control on the same days. The Oscillating treatment and the Positive Control treatment were compared at each milking. To determine if the MUN of cows on the Oscillating treatment ever was statistically similar to MUN concentration of cows on the Positive Control a third model was used: $Y = \mu + \text{cow (random effect, df = 20)} + \text{period (random effect, df = 2)} + \text{treatment (fixed effect, df = 1)} + \text{error (random effect, df = 18)}$. Treatments were Positive Control and Oscillating treatment.

Protein deficiency experiment

To determine how long (or if) cows would respond to the lowest CP diet fed (Oscillating-10.3), 8 additional Holstein multiparous dairy cows were moved into the tie stalls during period 2 and fed the control diet for 5 d. A priori, a decrease in milk yield of 5 kg/d by cows fed the low CP diet was set as the endpoint for this experiment or 10 d

whichever came first. Cows were managed the same as cows on the Latin square experiment.

Milk yields and feed offered and refused were recorded as described above. Real time statistical analysis (see below) was completed each day to determine whether or not the 10.3% CP diet reduced milk yield by 5 kg/d compared to the Positive Control. P.M. milk samples for the eight cows on the 10.3% CP diet and the seven cows on the Positive Control diet in period 2 were collected for 4 d (starting on the afternoon of the second day cows were fed the 10.3% CP diet). The P.M. milk samples were refrigerated and then assayed for MUN every 2 d as described above.

The model: $Y = \mu + \text{covariate} + \text{cow (random effect, df = 14)} + \text{treatment (fixed effect, df = 1)} + \text{error}_1 \text{ (random effect, df = 13)} + \text{day (fixed effect, df = 4)} + \text{treatment} \times \text{day (fixed effect, df = 6)} + \text{residual error}_2 \text{ (random effect, df = 23)}$ with day as a repeated measure was used in real time to analyze milk production of the 10.3% CP cows to determine when milk yield dropped 5 kg/d compared to the Positive Control cows. The covariate used in the equation was the average milk yield of each cow for the 6 d the cows were adjusted to the Positive Control before switching to the 10.3% CP diet. The same model (except no covariate and day df = 3) was used to compare MUN of the Positive Control cows versus the cows fed the 10.3% CP diet.

Method comparisons

DHI milk protein yields were used to determine NUE [(N from DHI true protein/0.94)/N Intake]; therefore, we wanted to determine if there was a difference in NUE if we used DHI results or KJ N results. To compare the infrared method used by

DHI to the KJ CP method used in our lab, the true protein results from d 25, 26, 27, and 28 from DHI were averaged by cow (not weighted by A.M./P.M. milk yield) and divided by 0.94 to obtain CP. CP was divided by 6.38 to obtain milk N. KJ CP values from composite samples of each cows on d 25 to 28 were also divided by 6.38 to obtain milk N. Milk N (from the 2 methods) was divided by N intake to obtain NUE. The NUE results by cow compared between methods using a 2-way analysis of variance with PROC MIXED using the model: $\text{NUE} = \text{treatment (df = 2)} + \text{cow (df = 20)} + \text{period (df = 1)} + \text{method (df = 1)} + \text{method} \times \text{treatment} + \text{residual error}_1 \text{ (df = 18)}$. The results were also compared using a paired t-test when the NUE by cow was paired. A regression of the DHI MUN method on our lab MUN method was performed using PROC REG. The regression coefficients were compared to 0 and 1 (intercept and slope, respectively). For the 4 d milk was collected and sent to DHI each period (d 25, 26, 27, and 28), the DHI MUN method and our lab's method (modified Stanbio kit) were compared statistically the same way as above except the model for PROC MIXED was: $\text{MUN} = \text{treatment (df = 2)} + \text{cow (period) (df = 20)} + \text{residual error}_1 \text{ (df = 39)} + \text{method (df = 1)} + \text{method} \times \text{treatment (df = 2)} + \text{day (df = 3)} + \text{treatment} \times \text{day (df = 6)} + \text{day} \times \text{method (df = 3)} + \text{day} \times \text{treatment} \times \text{method (df = 6)} + \text{residual error}_2 \text{ (df = 168)}$ with day as a repeated measure.

Chapter 4: Results and Discussion

Diet

The CP concentrations of the diets (Table 2) were close to the target values of 16.9, 13.6, and 10.3% CP for the Positive Control, Negative Control, and Oscillating-10.3 diets respectively. The estimated RDP and RUP concentration decreased as diet CP decreased because soybean meal was replaced with corn grain and soyhulls (Tables 1 and 2).

Based on Lammers et al. (1996), particle size for corn and alfalfa silages should have been adequate for saliva production (Table 3). The in vitro NDF digestibilities (IVNDFD) were 64, 69, and 71% of NDF for the Positive Control, Negative Control, and Oscillating-10.3 diets respectively (Table 2). NDF digestibility increased as more soyhulls were added to the diets and as the proportion of forage NDF (fNDF) to total NDF decreased. When soyhulls replaced corn silage in dairy cow rations, reducing fNDF from 18% to 12% (total NDF 36% and 39% of DM), in vitro digestibility of organic matter (OM) and NDF increased with the soyhull diet compared to the corn silage diet (Miron et al., 2003). When nonforage fiber sources such as soyhulls are added to diets with adequate fNDF, the coarse forage particles can increase the digestibility of the nonforage NDF by slowing down the passage rate of the soyhulls. In an alfalfa hay based diet with 25% of DM as soy hulls, the ruminal mat consistency increased by 49%, which

slowed the passage rate of the soyhulls by 16% (Grant, 1997). As the NSC content of the diets is reduced such as from the Positive Control to the Oscillation-10.3 diet, NDF digestibility may also increase due to greater digestibility of soyhull fiber or a decrease in negative associative effects (Sarwar et al., 1992). Studies have shown that readily digestible fiber from soyhulls is as effective as NSC from corn in providing fermentable energy for N digestion and synthesis of MCP as long as soyhulls are less than 30% of diet DM (Ipharraguerre et al., 2002; Ipharraguerre and Clark, 2003). With the increase in readily digestible fiber from soyhulls, there was a decrease in protein due to lower concentrations of soybean meal in the diets. The carbon from amino acids in protein can be a fermentable energy source. Therefore, the IVNDFD data suggest fermentable energy across treatment diets was likely similar.

Analytical method comparisons

Many NUE values in literature are reported from DHI-type true protein analyses; however, true protein does not account for the nonprotein N secreted into milk. Therefore, NUE based off of crude protein was used to calculate NUE. When NUE calculated by the milk protein infrared method used by DHI was compared to NUE calculated by the KJ CP method used in our lab, both the ANOVA analysis and paired t-test indicated that these methods are not comparable with each other when calculating NUE ($P < 0.0001$; Table 5). The equation for the regression line was: $\text{DHI} = 8.77 + 0.641\text{KJ}$. The intercept was not equal to 0 ($P < 0.0001$), and the slope was not equal to 1 ($P < 0.0001$). KJ can be more accurate in measuring milk protein as the infrared systems have to be re-calibrated for each set of samples, which can cause some inaccuracies

(O'Sullivan et al., 1999). NUE reported in Table 9 was calculated using DHI true proteins and dividing by 0.94 to obtain crude protein because composite samples were not made for period 1. Using DHI true protein to calculate NUE may underestimate actual efficiency because there is nonprotein N in milk. Even with NUE using milk N from the KJ method, treatments differences would have been similar.

When the MUN segmented flow analyzer method used by DHI and the modified Berthelot reaction method that was used in our lab were compared, both the ANOVA ($P < 0.0001$) and the paired t-test ($P < 0.0001$) indicated the methods yield different results (Table 6). However, the regression line equation was $\text{DHI} = 0.464 + 1.04\text{Lab}$ where the intercept is equal to 0 ($P = 0.03$), and the slope is equal to 1 ($P = 0.05$). Lima et al., (1998) reported that when comparing the flow injection system with the Boehringer UV test and the colorimetric assay used by Association of Official Analytical Chemists for analyzing MUN, the deviation was less than 5%. The flow injector system may be able to quantify the urea content within a wider concentration range compared to a colorimetric method similar to the one used in our lab (Lima et al., 1998). The MUN results reported were from a modified Berthelot reaction (a colorimetric assay); however, treatments differences would have been similar with results from DHI.

DMI, Milk Yield and Milk Composition

To determine when (or if) the 10.3% CP diet would be limiting in MP and reduce milk yield, the diet was fed to 8 cows continually for 5 d. Milk yield dropped from 34.0 kg/d on the first day the diet was fed to 28.6 kg/d on the second day the 10.3% CP diet was fed (Figure 7). Starting on the second day the 10.3% CP diet was fed, milk yield for

the cows fed the 10.3% CP diet was lower ($P = 0.03$) than the cows fed the Positive Control (16.4% CP), indicating that the diet was deficient in MP (Figure 7). Milk yield decreased for the cows fed the 10.3% CP diet due to a decrease in DMI ($P = 0.009$; Figure 8) for the cows fed the 10.3% CP diet compared to the Positive Control.

The Negative Control diet was formulated to be deficient in MP and to reduce milk yield compared to the Positive Control, but cows fed the two treatments were similar ($P = 0.31$) in milk yield. During the experiment, cows on the Positive Control averaged 35.4 kg/d of milk compared to the predicted MP-allowable milk of 45.4 kg/d. Cows on the Negative Control averaged 34.7 kg/d of milk compared to the predicted MP-allowable milk of 28.6 kg/d. The NRC could have over-predicted the protein requirements, and the Negative Control may not have been deficient in MP. Cows on average were producing 41 ± 7 kg/d milk when the diet was formulated and 38 ± 8 kg/d on average for the 5 d all cows were adjusted to the Positive Control. Milk yield decreases as lactation progresses, and on average milk yield decreased about 3 kg/d over a period for cows on all treatments (Figures 9 and 10). In long term 10-week studies, diets with 14% CP reduced milk production in cows producing 39 to 43 kg/d compared to cows fed diets with 15-16% CP due to decreased total tract NDF digestibility and DMI (Hristov and Giallongo, 2014). Huhtanen and Hetta (2012) reported that based on a meta-analysis of 204 studies, intake and milk production responses were not affected by experimental design of cross-over or continuous designs. However, cross-over designs did underestimate milk yield responses if there was a large variation in intake potential (Huhtanen and Hetta, 2012). Diets with 14% CP can decrease intake, but usually the

decrease in DMI and milk yield takes longer than 4 wk to occur (Hristov and Giallongo, 2014). Therefore, a difference between the Positive and Negative Controls in milk yield may have been observed if diets were fed for longer than 4 weeks, or the NRC over-predicted MP requirements.

Further, NE-allowable milk was lower than MP-allowable milk for the Positive Control (41.8 kg/d vs. 45.8 kg/d). On average, cows gained 26 kg each period (about 0.93 kg/d). The energy required for the added 0.93 kg/d of BW would be 4.5 Mcal/d. Since the predicted energy content of the milk for the Positive Control is 0.68 Mcal NE_L/ kg of milk, 4.5 Mcal/d for BW gain would equal about 6.6 kg/milk. With the energy required for BW gain taken into account, NE-milk is equal to (35.2 kg/d) the actual milk yield of 35.4 kg/d for the Positive Control. Also, given that book values were used for lignin and other variables used to measure the energy content of feeds, diets may have had a lower energy content than predicted by the NRC.

Recommendation for dietary starch content for lactating dairy cows is commonly between 23% and 30% DM (Dann and Grant, 2009). When 8 ruminally and duodenally cannulated cows were fed either ground high-moisture corn or dry ground corn at two dietary levels of starch (32% or 21% DM), cows fed the high starch diets had greater milk yields compared to cows fed the low starch diet regardless of corn grain type (Oba and Allen, 2003a). Ruminal starch digestion depends on both starch concentration and diet fermentability. Dry ground corn reduced rumen fermentation and increased DMI when the dietary starch concentration was high (Oba and Allen, 2003a). Milk production was higher for cows fed the dry ground corn diet compared to cows fed the high-moisture

corn diet at the high concentration of dietary starch due to an increase in DMI; however, corn grain type did not affect milk production when dietary starch levels were low (Oba and Allen, 2003a). The ruminal starch digestibility was greater between the corn grain types when the dietary starch concentration was high compared to low (Oba and Allen, 2003b). Ruminal starch digestion is limited by enzyme activity and starch concentration; therefore, the contents of the rumen may not have contained enough amylase for maximal starch digestion when diets were low in starch, resulting in similar starch digestibilities between the two corn grain types (Oba and Allen, 2003b). IVNDFD was lowest for the Positive Control diet, and along with the low concentration of starch in the diet, fermentable energy from the Positive Control diet have been limiting as MP-allowable milk was higher than actual milk production and milk protein and lactose concentrations were low.

There was a trend for energy-corrected milk (ECM; $P = 0.11$), milk fat yield ($P = 0.11$), and milk protein yield ($P = 0.11$) to be lower for the Oscillating treatment compared to the Negative Control (Table 7). The decrease in ECM would either be due a decrease in DMI or less efficiency of nutrient utilization for the Oscillating cows. However, cows fed the Oscillating treatment and cows fed the Negative Control had similar milk yields ($P = 0.15$) and DMI ($P = 0.16$). There was a treatment x day effect ($P < 0.0001$) for the Oscillating treatment milk yield (Table 8). Milk yield decreased ($P < 0.0001$) from the first day cows on the Oscillating treatment were fed the Oscillating-10.3 diet to the second day they were fed the Oscillating-10.3 diet. Therefore, the trend for a decrease in milk component yields for the Oscillating cows compared to those fed the

Negative Control may have been due to the decline in milk yield on the second day the cows on the Oscillating treatment were fed the Oscillating-10.3% CP diet. Milk protein yield could have decreased with the 10.3% CP diet because it was limiting in MP. In a previous experiment, milk protein yield was lower for cows fed a diet with 11.3% CP compared to cows fed a diet with 15.4% CP (Metcalf et al., 1996). Further, the concentration of K was lower in the 10.3% CP diet, and a low K has been seen to decrease milk fat yield. Increasing K from 0.9 to 1.7% of DM increased 3.5% FCM although the effect was dependent on chloride as well (Sanchez et al., 1994). With regard to milk component yields, the Oscillating treatment had a negative impact.

The MUN concentration also decreased ($P < 0.0001$ on d 2) for the cows fed the constant 10.3% diet compared to the Positive Control by the second day, indicating that the 10.3% CP diet is lower than the Positive Control in CP (Figure 11). The MUN concentration for cows fed the Positive Control was higher ($P < 0.0001$) than the cows fed the Negative Control (Table 7). The concentration of MUN for the Positive Control is similar to those reported in other studies. Cows fed a diet with 16.7% CP had an average MUN concentration of 12.4 mg/dL (Broderick, 2003). Cows fed a diet with 16.8% CP and 7.7% RUP had an average MUN concentration of 14.3 mg/dL (Davidson et al., 2003). The MUN concentration for the Negative Control was also within the range of other studies. When cows were fed a diet with 13.5% CP, the average MUN concentration was 7.7 mg/dL (Brito and Broderick, 2006). When cows were fed a 13.6% CP diet, the average MUN was 10.3 mg/dL (Lee et al., 2012a).

On average, the cows fed the Oscillating treatment had the same MUN ($P = 0.38$) as cows on the Negative Control (Table 7) except that MUN followed a cyclic pattern for Oscillating cows (Figure 12). MUN concentration may have been similar for the Negative Control and the Oscillating treatment because cows were being fed, on average, the same amount of protein. When steers were fed diets of 11% CP, 13% CP, 15% CP, or a treatment where CP concentration oscillated every 2 d between the 11% and 15% CP diets, steers on the Oscillating treatment had a lower mean serum urea nitrogen (SUN) concentration than those steers fed the 13% CP diet (Ludden et al., 2003). Steers on the Oscillating treatment fed the 11% had an average SUN concentration similar to those fed the constant 11% CP diet. Steers on the Oscillating treatment fed the 15% CP diet had an average SUN concentration similar to those on the constant 13% CP diet (Ludden et al., 2003). The lower SUN for steers on the Oscillating treatment fed the 15% CP diet compared to the steers fed the constant 15% CP diet suggests that steers on the Oscillating treatment fed the 15% CP diet were more efficient in utilizing protein than those steers fed the constant 15% CP diet and could be why the steers on the Oscillating treatment overall had a lower SUN than steers on the 13% CP diet. Therefore, average MUN may have been similar for cows fed the Negative Control and the Oscillating treatment because NUE for both treatments was similar (Table 9; $P = 0.31$).

Average MUN concentrations rapidly changed after diets were switched (Figure 13). Within 15 hours MUN increased by about 40% when Oscillating cows were switched to the Positive Control, and average MUN dropped by almost 70% within 12 hours after Oscillating cows were fed the Oscillating-10.3 diet (Figure 13). However, on

average, cows on the Oscillating treatment when fed the Positive Control for 2 d had a lower ($P = 0.01$) MUN compared to cows fed the constant Positive Control treatment fed for 2 d (Figure 13; 13.5 mg/dL vs. 14.6 mg/dL for the Oscillating cows when fed Positive Control and cows fed the Positive Control constantly). With the Oscillating treatment MUN did not reach the maximum dietary response, which means that BUN levels may not have reached concentrations as high as the Positive Control. If BUN concentrations did not reach the maximum dietary response, then urea recycling to the rumen may not have been enough to buffer the effects of feeding the deficient MP diet (10.3% CP), resulting in similar milk yields and milk protein yields for the cows fed the Oscillating treatment and cows fed the Negative Control. Oscillation may stimulate the transfer of N from one segment of the gut to another (Cole, 1999). For instance, if the rumen is deficient in protein after being fed an MP deficient diet, but the large intestine still has an excess of N from the days the animals were fed a MP adequate diet, then N could be absorbed from the large intestine and recycled back to the rumen (Cole, 1999). Because MUN concentrations for cows fed the Oscillating treatment did not reach the maximum dietary response, then BUN levels may not have been sustained and not as much excess N may have been recycled back to the rumen.

N intake and output

N intake increased as dietary CP concentration increased as expected (Table 9). The NUE efficiency increased from 27.3% to 32.8% as CP decreased from 16.4% CP to 13.4% CP. Broderick (2003) reported that NUE increased from 23.9% to 30.3% as CP in the diet decreased from 18.4% CP to 15.1% CP. As CP% increases, RDP% of the diet

will usually increase, causing a higher rumen ammonia concentration, and not all of the ammonia will be captured by microbes to make MCP; therefore, the excess ammonia will be converted to urea in the liver. Much of that urea will be excreted into the urine, decreasing NUE. If too much indigestible RUP is fed cows, then NUE will also decline as CP% increases. Diets with a high amount of digestible RUP will usually increase NUE if diets are insufficient in MP and RDP (Noftsger and St-Pierre, 2003; Kalscheur et al., 2006). Digested RUP in the form of urea can be recycled back to the rumen and be used to synthesize MCP, masking the deficiency in RDP (Kalscheur et al., 2006). If RDP is adequate, then amino acids from excess RUP will be catabolized, and the N will be excreted into the manure, decreasing NUE. N intake and NUE were similar for the Oscillating treatment compared to the Negative Control, which does not support the hypothesis that oscillation would increase NUE compared to the Negative Control.

The MUN method of estimating urinary N excretion (Kauffman and St-Pierre, 2001) is dependent on accurate body weights. Body weights were recorded on d 28 of each period, and urine was collected on d 21-24; therefore, UN excretion should have been accurate according to body weight. In lambs, urinary N excretion decreased by 20% within 24 h of switching from an adequate CP diet to a deficient CP diet (Liu et al., 1995). After 2 d of being fed a reduced CP diet, urinary excretion decreased by 29% (Liu et al., 1995). In the present trial, MUN showed a trend to increase ($P = 0.07$) from the first d to second d the Oscillating cows were fed the Positive Control, and decreased ($P = 0.01$) from the first d to second d the Oscillating cows were fed the Oscillating-10.3 diet. Therefore, predicting UN excretion from MUN may have been accurate, although the

method has not been used when dietary CP concentrations oscillate. For the creatinine method in estimating urine N, bilirubin and other hepatic metabolites can negatively interfere with the creatinine assay, causing creatinine assay results to be low (Bowers and Wong, 1980). Bilirubin is the main compound that interferes with the assay with an estimated decrease in creatinine of 1 mg per 100 mg of bilirubin. When creatinine assay results have been found to be lower than normal, the decrease in creatinine assay results is higher than can be accounted for with just bilirubin interference; therefore, Bowers and Wong (1980) suggested that other hepatic metabolites or other compounds may also interfere with the creatinine assay. Glucose and acetoacetate have also been known to interfere with creatinine assays (Marakala et al., 2012). The urine samples were stored and frozen for about 6 months. The samples were stored in plastic bottles that had been used before and washed. Therefore, there could have been a compound from the samples previously stored in the bottle or from the plastic that interfered with the creatinine assay results. Because the creatinine assay results were low, average urine excretion of cows by the creatinine method was 44 L/d (Table 9), which is higher than urine volumes measured by Weiss and Wyatt (2006) and Weiss et al. (2009b) over a range of dietary CP concentrations. Urine N as % of intake estimated from the creatinine assay results was also higher than urine N estimated by Broderick (2003) using the creatinine method and urine N estimated by Davidson et al. (2003) using the MUN method. Therefore, N excretions estimated by the creatinine method are reported in Table 9 but are not discussed further.

Urine excretion was also estimated using the UN excretions estimated by MUN (Kauffman and St-Pierre, 2001) and KJ N of spot urine samples. Estimated urine excretion using the UN excretions estimated by MUN increased ($P < 0.0001$) as the diets increased from 13.4% CP to 16.4% CP (Table 9). Urine excretion for the Oscillating treatment and the Negative Control were similar ($P = 0.36$) as CP was similar in the diets on average. With an increase in CP concentration of the diet there will be more N metabolites excreted in the urine, requiring more water in the urine to dilute the metabolites (Valadares et al., 1999). Average estimated urine excretion was similar to urine excretion reported by Wattiaux and Karg (2004) but higher than measured urine excretion reported in Weiss et al., (2009b) and Weiss and Wyatt (2006). As CP concentration in the diet increased, urine N excretion also increased as a % of N intake ($P < 0.0001$; Table 9). As a % of N intake, UN for the Positive Control was higher than the average UN of 32 or 33% of N intake reported by Kauffman and St-Pierre (2001) and Wattiaux and Karg (2004) with diets similar to the Positive Control. The differences in UN as % of intake could have been due to the differences in RDP and RUP concentrations or N intake of the Positive Control compared to those diets in Kauffman and St-Pierre (2001) and Wattiaux and Karg (2004). Increasing CP% of the diet usually increases rumen ammonia assuming that more degradable protein is added to increase dietary CP, and not all of the rumen ammonia will be captured by microbes to form MCP. Therefore, N from any excess ammonia, as well as N from catabolized amino acids will be excreted into the urine.

Average UN excretion as a % of N intake was similar ($P = 0.90$) for cows fed the Oscillating treatment and cows fed the Negative Control. When lambs were fed diets with 13% CP, 15% CP, 17% CP, or a treatment where the dietary CP concentration oscillated between 13% CP and 17% CP, urinary N excretion increased from 48 to 57 % of N intake as CP concentration increased from 13% CP to 17% CP for the constant treatments (Ludden et al., 2002). UN excretion was similar for the lambs on the Oscillating treatment and those on the constant 13% CP diet. The same trends were found when steers were fed diets with 12% CP, 14% CP, or a treatment where the dietary CP concentration switched between 12% CP and 14% CP every 2 d (Cole et al., 2003).

Estimated fecal N excretion as a % of N intake and fecal N (g/d) increased ($P = 0.03$) as CP decreased from 16.4% CP to 13.4% CP (Table 9). The increase in apparent N digestibility when cows were fed the Positive Control compared to the Negative Control may have been due to the greater true digestibility of protein in the 16.4% CP diet or could have been a result of a dilution of metabolic fecal N (Kauffman and St-Pierre, 2001). Fecal N as % intake and fecal N (g/d) were similar ($P > 0.65$) for cows fed the Oscillating treatment and cows fed the Negative Control. When lambs were fed diets with 10% CP, 12.5% CP, 15% CP or a treatment where the CP concentration of the diet oscillated between 10% CP and 15% CP every 2 d, fecal N excretion (g/d) increased as dietary CP concentration increased, and the lambs fed the Oscillating treatment had similar fecal N excretions (g/d) as lambs fed the continuous 12.5% CP diet (Cole, 1999). In a similar study with sheep, fecal N excretion remained the same among treatments as CP increased from 13% CP to 17% CP (Ludden et al., 2002). Therefore, in our trial with

dairy cows and other experiments with sheep and cattle, N excretion increases as N intake increases.

When steers were fed diets of 9% CP, 12% CP, 14% CP or a treatment that oscillated between 9% and 14% CP every 48 h, N retention was greater for the steers fed the Oscillating treatment compared to the steers on the 9% CP and 14% diets (Archibeque et al., 2007). When lambs were fed diets of 11.5% CP, 13.7% CP, 17.6% CP or a treatment oscillating between 11.5% and 17.6% CP, the N retention was higher for lambs on the Oscillating treatment than those fed the 13.7% CP diet (Doranalli et al., 2011). Doranalli, et al. (2011) suggested that the Oscillating treatment may have increased N retention through increased urea recycling, as indicated by a higher estimated microbial N supply for the lambs fed the Oscillating treatment compared to those fed the 13.7% CP diet. In the present trial, estimated urine and fecal N as % of N intake did not decrease for the cows fed the Oscillating treatment compared to those fed the Negative Control. When cows on the Oscillating treatment were fed the 10.3% CP diet, milk yield decreased by the second day. Therefore, 2 d may have been too long for the oscillation half-cycles. Feeding the 10.3% CP diet every other day or feeding the Positive control 2 d and the 10.3% CP diet 1 d may have produced different results, especially since 2 d on the 10.3% diet decreased ECM.

Conclusions

Milk yield was not different for cows fed the Positive Control and the Negative Control possibly because of study design or the NRC over-estimated MP requirements. The Positive Control was lowest of the diets in starch content and IVNDFD, suggesting

that fermentable energy may have been limiting even though CP% was highest among diets, especially since milk protein and lactose concentrations were lower than typical for the average Holstein. Because milk yield was similar for cows fed the Positive Control and Negative Control, the 13.4% CP treatments (Negative Control and Oscillating treatment) may have met the MP requirements of the cows; therefore, interpretation of whether or not oscillation would improve milk yield, milk protein yield or NUE over a diet limited in protein is restricted. Oscillation did not decrease milk yield compared to the Negative Control. However, oscillation negatively impacted or at most maintained ECM, and milk protein and fat yields compared to the Negative Control. The negative impacts of the Oscillating treatment and the rapid decrease in milk yield when cows were constantly fed the 10.3% CP diet indicates that 2 d may have been too long for the oscillation half-cycles. The negative impacts of oscillation also indicate that because N requirements for growing lambs and steers differ from N requirements of dairy cows, oscillation may not increase N retention in dairy cows as it does in growing ruminants. Overall, 2 d oscillations of a MP deficient diet and a diet that met MP requirements did not improve milk and milk protein yields nor did it increase NUE over a diet formulated to be deficient in MP.

Table 1: Ingredient composition of diets, (% of DM)

Ingredient	Diet ¹		
	Positive Control	Negative Control	Oscillating-10.3
Corn silage	50.0	50.0	50.0
Alfalfa silage	10.0	10.0	10.0
Concentrate mix	40.0	40.0	40.0
Soybean hulls	12.2	18.4	24.6
Aminoplus ²	10.6	5.3	-
Soybean meal, 48% CP	7.4	3.7	-
Corn grain, dry, ground	4.9	7.4	9.9
Urea	-	0.14	0.28
Animal-vegetable fat	1.05	1.05	1.04
Limestone	1.05	0.88	0.72
Sodium bicarbonate	0.84	0.84	0.84
TM salt	0.68	0.68	0.68
Dicalcium phosphate	0.44	0.66	0.88
Biotin, 220 mg/kg	0.36	0.36	0.36
Selenium, 198 mg/kg	0.15	0.15	0.15
Vitamin E, (44 IU/g)	0.08	0.08	0.08
Vitamin D, (3000 IU/g)	0.04	0.04	0.04
Vitamin A, (30,000 IU/g)	0.01	0.01	0.01
Dynamate ³	-	0.18	0.36
Zinpro 120 ⁴	0.002	0.002	0.002
Copper Sulfate (5H ₂ O)	0.002	0.002	0.002

¹ Diets were Positive Control (16.4% CP), Negative Control (13.4% CP), and Oscillating-10.3% CP. The Oscillating treatment consisted of the 10.3% CP fed for 2 d followed by the Positive Control diet fed for 2 d repeated over the 28 d period to average 13.4% CP.

² Ag Processing Inc. (Omaha, NE).

³ Mosaic (Plymouth, MN).

⁴ Zinpro (Eden Prairie, MN).

Table 2: Nutrient composition of diets (DM basis)

Nutrient	Diet ¹		
	Positive Control	Negative Control	Oscillating-10.3
DM%	60.0	60.0	60.0
CP ²	16.4	13.4	10.3
RDP ³	10.0	8.6	7.2
RUP ³	6.5	4.8	3.1
Starch	20.4	22.0	23.6
NDF	34.5	37.8	41.1
Forage NDF	23.1	23.1	23.1
Ash	6.8	6.7	6.6
Na	0.59	0.57	0.55
Mg	0.22	0.23	0.25
Ca	1.14	1.16	1.17
P	0.39	0.42	0.44
30 hr. IVNDFD, % NDF ⁴	63.8	69.2	71.2
MP allowable milk ³	45.4	28.6	17.0
NE _L , Mcal/kg ³	1.45	1.48	1.41

¹ Treatments were Positive Control (16.4% CP), Negative Control (13.4% CP), and Oscillating-10.3% CP. The Oscillating treatment consisted of the 10.3% CP diet fed for 2 d followed by the Positive Control diet fed for 2 days repeated over the 28 d period to overall average 13.4% CP.

² Calculated from Kjeldahl CP of feeds.

³ NRC (2001) using actual DMI.

⁴ 30-hr In vitro NDF Digestibility; Cumberland Valley Analytical Services (Hagerstown, MD); N = 6.

Table 3: Nutrient composition of corn and alfalfa silage (% of DM)

Nutrient	Corn Silage		Alfalfa Silage	
	Average	SD	Average	SD
DM ¹	41.1	1.8	37.1	4.9
Starch ²	31.9	2.4	ND	ND
NDF ²	37.2	1.5	43.9	2.9
CP ¹	7.3	0.3	18.3	1.9
Ash ²	3.6	0.3	8.6	1.0
Particle size, % as fed ³				
Upper sieve	7.0	2.7	28.2	2.7
Lower sieve	69.0	4.5	46.0	4.5
Bottom pan	24.0	2.3	25.8	4.1

¹ n = 24.² n = 6.³ Penn State Particle Separator (Upper sieve .75 inches; Lower sieve .31 inches).**Table 4:** Nutrient composition of Positive Control and Oscillating-10.3 concentrate mixes (% of DM; n = 6)

Nutrient	Positive Control ¹		Oscillating-10.3 ²	
	Average	SD	Average	SD
Starch	10.0	1.1	18.1	3.1
NDF	28.9	1.8	45.3	2.8
CP	27.4	1.0	12.1	1.4
Ash	10.3	0.4	10.0	0.3

¹ Positive Control treatment (16.4% CP).² Oscillating treatment consisted of a Oscillating-10.3 diet (10.3% CP) fed for 2 d. followed by the Positive Control fed for 2 days repeated over the 28 d period to average 13.4% CP.

Table 5: NUE calculated by using Milk N from DHI or KJ

Treatment ³	DHI NUE ¹		KJ NUE ²	
	Average	SEM	Average	SEM
Positive Control	25.1 ^a	1.64	26.6 ^a	1.64
Negative Control	32.2 ^a	1.67	36.6 ^b	1.67
Oscillating	29.8 ^a	1.48	32.8 ^b	1.48

¹ Nitrogen use efficiency (NUE; Milk N/ N Intake) calculated using Milk N from DHI infrared method.

² Nitrogen use efficiency (NUE; Milk N/ N Intake) calculated using Milk N from Kjeldahl (KJ) N method.

³ Positive Control- 16.4% CP; Negative Control-13.4% CP; Oscillating- 10.3% CP diet fed for 2 d followed by the Positive Control fed for 2 d repeated over the period to average 13.4% CP .

^{a-b} different letters P < 0.05 between methods.

Table 6: MUN from DHI or modified Berthelot reaction

Treatment ³	DHI MUN ¹		Lab MUN ²	
	Average	SEM	Average	SEM
Positive Control	15.3 ^a	0.26	14.1 ^b	0.24
Negative Control	9.1 ^a	0.26	8.4 ^b	0.24
Oscillating	9.0 ^b	0.26	8.5 ^b	0.24

¹ MUN (mg/dL) from DHI segmented flow analyzer method.

² MUN (mg/dL) from our lab's modified Berthelot reaction (STANBIO Laboratory, Enzymatic Urea Nitrogen Proc. No. 2050, Boerne) for periods 2 and 3 only.

³ Positive Control- 16.4% CP; Negative Control-13.4% CP; Oscillating- 10.3% CP diet fed for 2 d followed by the Positive Control fed for 2 d repeated over the period to average 13.4% CP.

^{a-b} different letters P < 0.05 between methods.

Table 7: DMI, milk production, and milk composition period averages

	Treatments ¹				P <	
	Positive Control	Negative Control	Oscil.	SEM	PC vs NC ²	Osc vs NC ³
BW, kg	690	683	690	7.6	0.10	0.13
BW change, kg ⁴	26.1	19.5	25.7	7.6	0.10	0.13
DMI, kg/d	25.3	25.1	24.7	0.40	0.24	0.16
Milk Yield, kg/d	35.4	34.7	33.8	2.5	0.31	0.15
ECM ⁵ , kg/d	33.9	33.6	31.0	0.86	0.86	0.11
Feed Efficiency ⁶	1.34	1.33	0.08	0.67	0.67	0.25
Lactose, %	4.72	4.67	4.70	0.06	0.15	0.38
Lactose, kg/d	1.67	1.63	1.60	0.15	0.30	0.31
Milk fat,%	3.37	3.46	3.41	0.11	0.07	0.33
Milk fat, kg/d	1.16	1.15	1.12	0.08	0.86	0.11
Milk protein,%	2.93	2.91	2.91	0.06	0.13	0.91
Milk protein, kg/d	1.00	0.99	0.96	0.06	0.42	0.11
MUN, mg/dL	14.5	9.05	8.95	0.34	<0.0001	0.58

¹Treatments: Positive Control (16.4% CP); Negative Control (13.4% CP); Oscillating-10.3% CP diet fed for 2 d followed by the Positive Control diet fed for 2 d repeated over the period to average 13.4% CP.

² PC vs NC: contrast for Positive Control versus Negative Control.

³ Osc vs NC: contrast for oscillating versus Negative Control.

⁴ BW change = |BW day 28 – BW day 28 previous period|.

⁵ ECM: energy-corrected milk calculated as ECM = (0.323 x kg milk) + (12.82 x kg milk fat) + (7.13 x kg milk protein) (DRMS, 2014).

⁶ Feed efficiency = ECM/DMI.

Table 8: Milk components by day for oscillating cows

	Days ¹					P <		
	H1	H2	L1	L2	SEM	Treatment*Day ²	H1 vs H2	L1 vs L2
DMI, kg/d	24.9	25.1	24.3	24.5	0.38	0.07	0.28	0.31
Milk yield, kg/d	32.1	35.4	35.6	32.2	0.51	<0.001	<0.0001	<0.0001
Lactose, %	4.71	4.70	4.69	4.69	0.06	0.95	NA ³	NA
Milk fat, %	3.39	3.19	3.48	3.61	0.12	0.004	0.05	0.18
Milk protein, %	2.89	2.94	2.91	2.90	0.07	0.07	0.004	0.48
MUN, mg/dL	9.05	10.0	9.04	7.69	0.34	<0.0001	0.07	0.01

¹Data from cows on Oscillating treatment only. H1 and H2 are the first and second day respectively that cows on the Oscillating treatment were fed the Positive Control (16.4% CP); L1 and L2 are the first and second day respectively that cows on the Oscillating treatment were fed 10.3% CP diet.

² There was no treatment by day effect for the two controls ($P > 0.10$).

³ Treatment by day effect was not significant ($P > 0.10$).

Table 9: N intake and outputs

	Treatments ¹				P <	
	Positive Control	Negative Control	Oscil.	SEM	PC vs NC ²	Osc vs NC ³
N intake ⁴ , g/d	628	486	497	12.6	<0.0001	0.39
Milk N ⁵ , g/d	166	163	159	5.60	0.32	0.13
NUE,% ⁶	27.3	32.8	31.9	1.11	<0.0001	0.31
MUN method ⁷						
Estimated urine excretion ⁴ , L/d	32.7	23.5	25.3	1.52	<0.0001	0.36
UN, g/d	250	150	155	6.17	<0.0001	0.33
UN, % intake	40.4	31.2	31.3	1.15	<0.001	0.90
Fecal N ⁸ , g/d	204	178	183	11.1	0.08	0.65
Fecal N, % intake	32.2	36.3	36.7	1.51	0.03	0.78
Creatinine Method ⁹						
Estimated urine excretion, L/d	48.5	39.7	39.1	2.80	0.01	0.87
UN, g/d	357	241	275	15.5	0.001	0.28
UN, % intake	63.9	51.6	55.1	5.22	0.09	0.55
Fecal N ⁸ , g/d	64.6	70.2	63.1	30.6	0.89	0.82
Fecal N, % intake	9.82	14.8	12.9	5.49	0.47	0.73

¹Treatments: Positive Control- 16.4% CP; Negative Control-13.4% CP; Oscillating- 10.3% CP diet fed for 2 d followed by the Positive Control diet fed for 2 d repeated over the period to average 13.4% CP.

² PC vs NC: contrast for Positive Control versus Negative Control.

³ Osc vs NC: contrast for oscillating versus Negative Control.

⁴ N Intake = (DMI x %N diet) – (Refusal x % N refusal).

⁵ Milk N = (DHI milk true protein yield/ 0.94)/ 6.38.

⁶ NUE = Milk N/ N Intake.

⁷ Urinary nitrogen = 0.0259 x BW (kg) x MUN (mg/dL); Urine excretion (mL/d) = UN (mg/d)/ Kjeldahl N (mg/mL).

⁸ Fecal N = N Intake – [Milk N + UN].

⁹ Urinary nitrogen = [(29 x BW (kg))/ creatinine (mg/mL)] x %N urine.

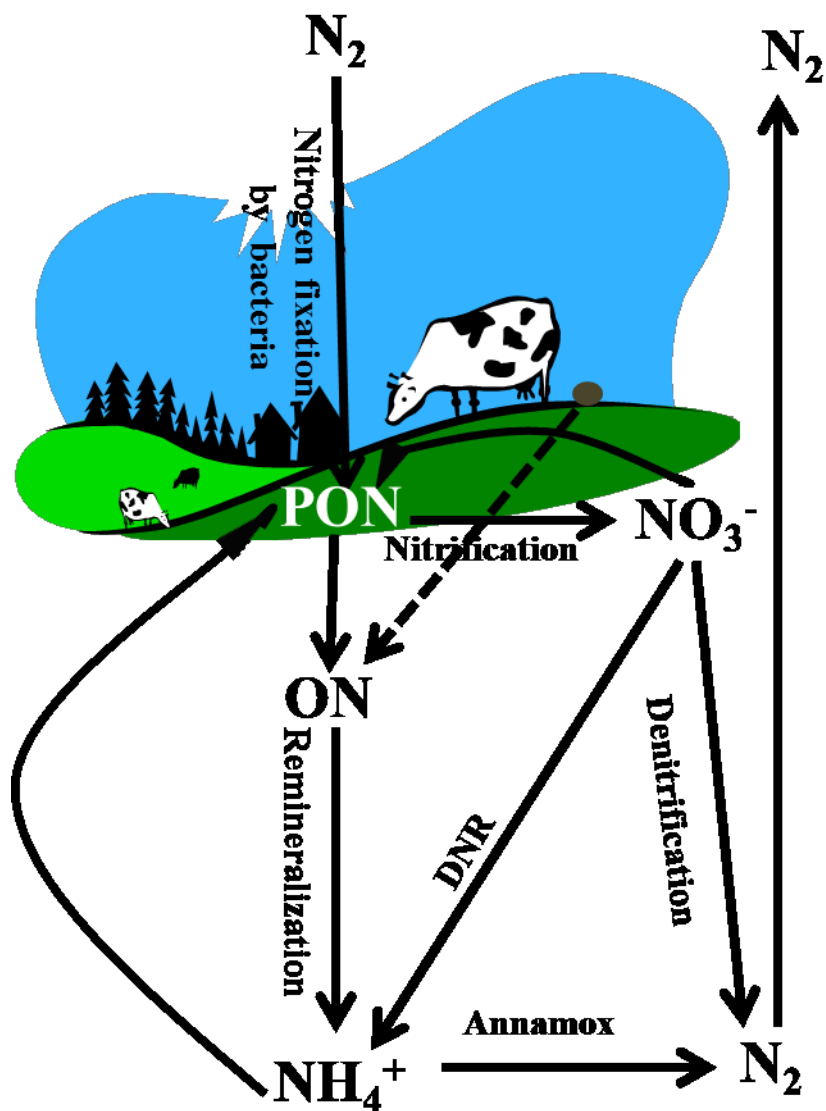


Figure 1: The nitrogen cycle (PON = plant organic nitrogen from symbiotic nitrogen fixing bacteria or from the soil; ON = organic nitrogen; DNR = dissimilatory nitrate reduction).

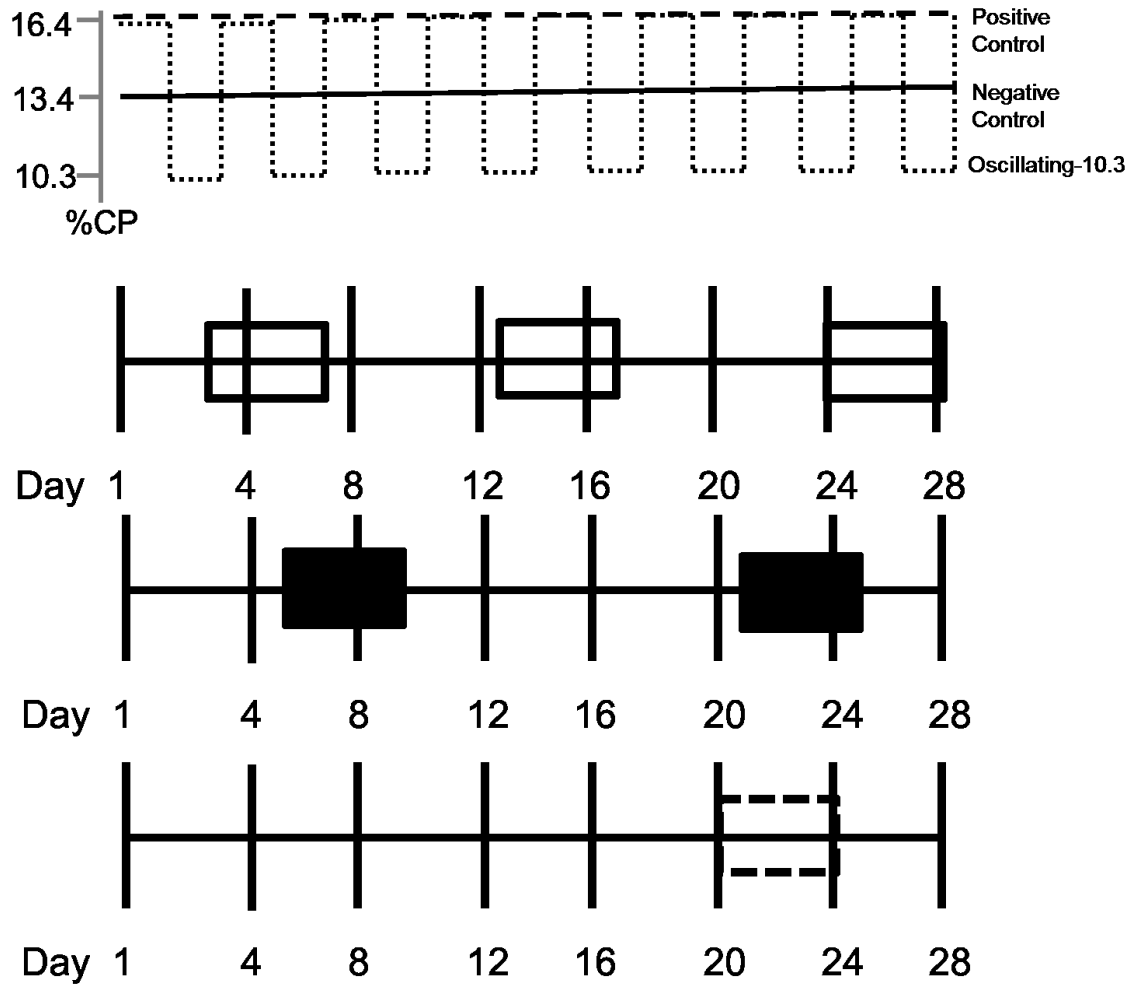


Figure 2: Treatment design for experiment. One full oscillation cycle is 4 d (between each of the black vertical bars with 7 full cycles per period; Cows on the Oscillating treatment were fed the Oscillating-10.3 for 2 d and Positive Control for 2 d). Milk samples (clear boxes with solid outline) were taken for three full oscillation cycles per period, refusal samples (solid boxes) were taken twice per period, and urine samples (clear boxes with dashed outline) were collected once per period.













Sun.	Mon.	Tues.	Wed.	Thurs.	Fri.	Sat.
	 	 DM started		  KJ N	 DM started	
Sun.	Mon.	Tues.	Wed.	Thurs.	Fri.	Sat.
	 	 DM started		  KJ N	 DM started	

Figure 3: Sampling schedule of feeds (only half of one period is shown; the schedule was repeated each period). Triangles represent when silages were sampled. The two triangles of the same type represent each 2 d composite silage sample analyzed for DM on the second day and KJ every Thursday (eight 2-d silage samples per period). All silage samples (triangles) were also composited into 2 wk samples (two 2-wk samples per period). The circles represent when concentrate was sampled at the barn and composited into 2 wk samples (two 2 wk samples per period). Concentrates were analyzed for Kjeldahl (KJ) N every other Thursday or whenever a new batch sample was taken from the feed mill.

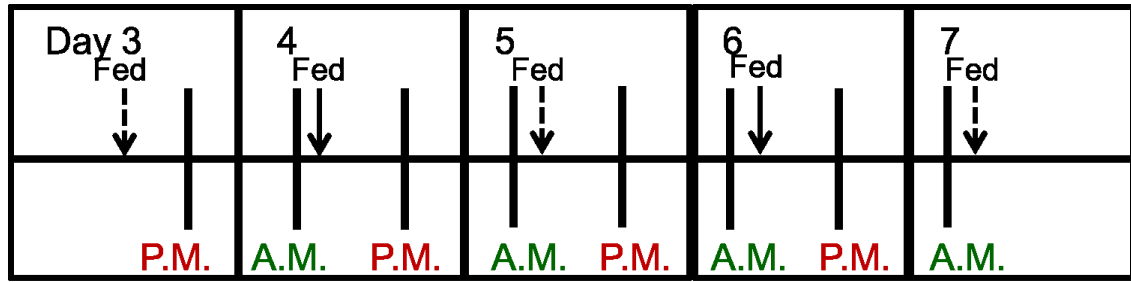


Figure 4: Milk sampling for first full oscillation cycle of each period. Sampling started at the P.M. milking (first milking after treatment diets were fed) on the first day of each oscillation cycle sampled and ended with the A.M. milking on the last day of the oscillation cycle samples. Therefore, for the first full oscillation cycle: milk was sampled at the P.M. milking on d 3, the A.M. and P.M. milkings on d 4,5, and 6, and the A.M. milking on d 7. The other two full oscillation cycles when milk was sampled each period followed the same sampling schedule as the first cycle shown above. Dashed arrows represent when diet was changed for the cows on the Oscillating treatment.

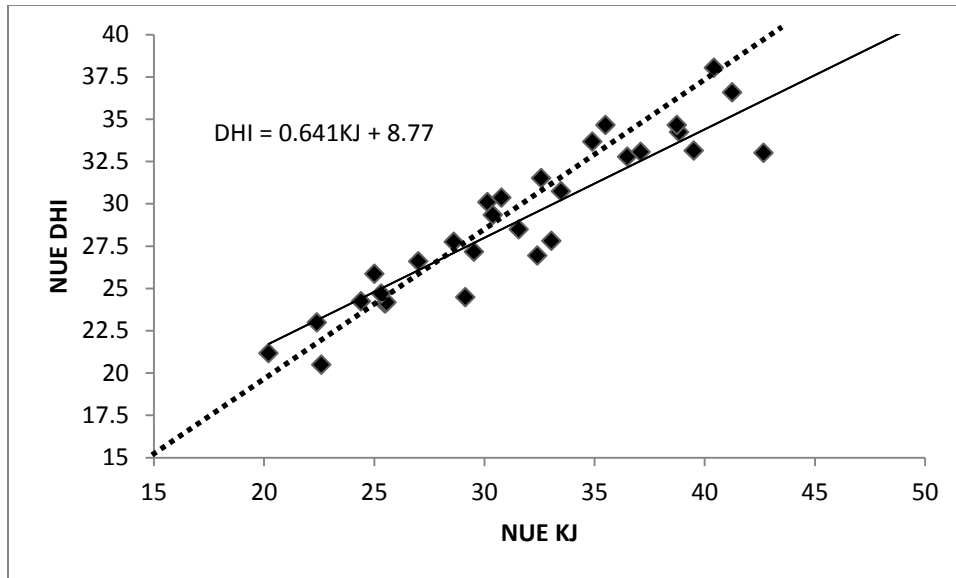


Figure 5: Regression of DHI infrared milk protein method and Kjeldahl (KJ) method for Nitrogen use efficiency (NUE). Each data point represents NUE (Milk N/N Intake) of each cow for the last 2 periods. NUE were calculated using the average of the true protein results from d 25, 26, 27, and 28 from DHI and divided by 0.94 to obtain DHI CP. The DHI CP and KJ CP values from composite samples of each cow on d 25 to 28 were divided by 6.38 to obtain Milk N. The intercept was not equal to 0 ($P < 0.01$), and the slope was not equal to 1 ($P < 0.01$), indicating that one method will not predict the other. The dashed line is the unity line $Y = X$.

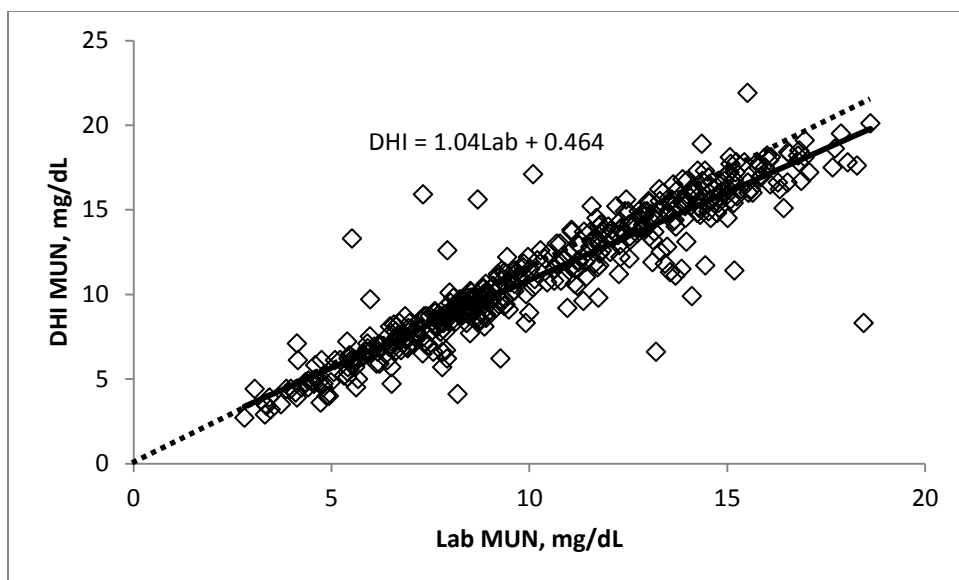


Figure 6: Regression of DHI flow analyzer MUN method and Lab method. Each data point represents the MUN concentration for each cow for days 25-28 in each period. MUN concentrations were measured using a Skalar SAN Plus segmented flow analyzer (Skalar Inc., Norcross, GA) by DHI Cooperative, Inc. (Columbus OH). MUN concentration were also measured after milk samples were filtered through 0.45 μ m syringe filters using a modified Berhelot reaction in our lab (BUN kit STANBIO Laboratory, Boerne, TX). The intercept is equal to 0 ($P = 0.03$), and the slope is equal to 1 ($P = 0.05$). The dashed line is the unity line $Y = X$.

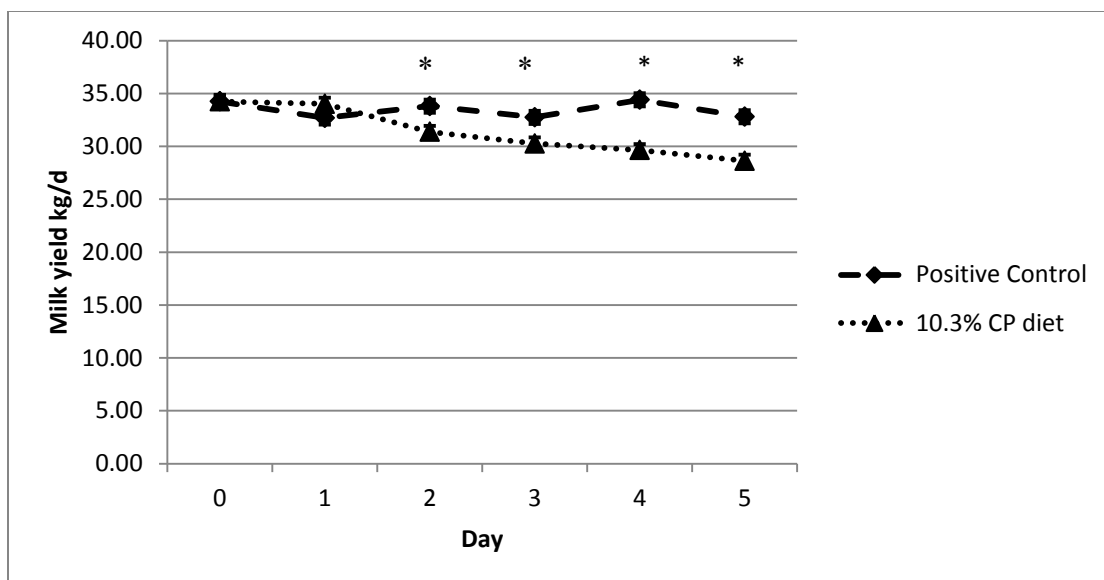


Figure 7: Milk yield for cows fed Positive Control versus the 10.3% CP diet for 5 d. Day 0 is average for 5 d all cows were fed the Positive Control. Milk yield for cows on the 10.3% CP diet was lower ($P = 0.03$) than milk yield for cows fed the Positive Control starting on d 2. * = $P < 0.05$ between treatments.

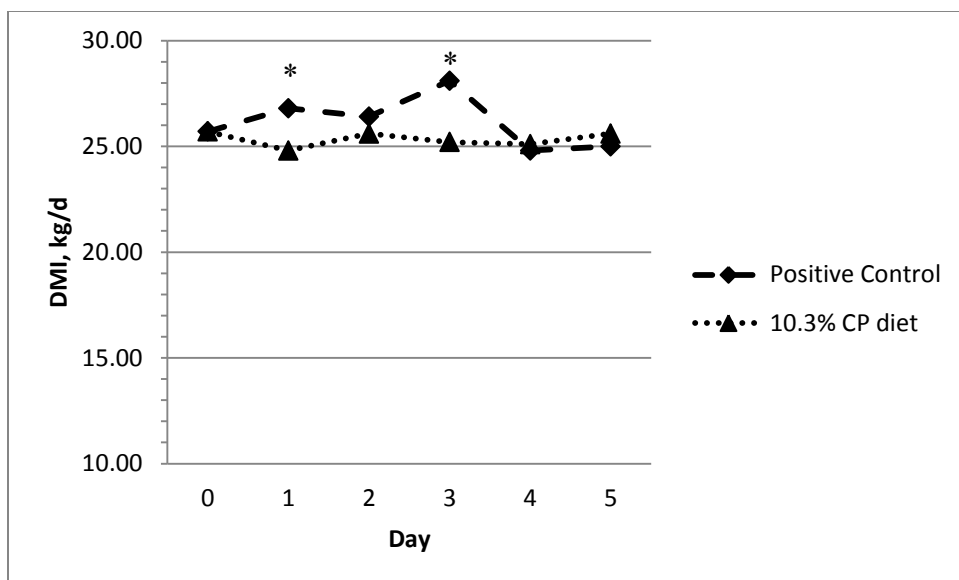


Figure 8: DMI for cows fed Positive Control versus the 10.3% CP diet for 5 d. Day 0 is average for 5 d all cows were fed the Positive Control. DMI for cows on the 10.3% CP diet was lower on average ($P = 0.009$) than DMI for cows on the Positive Control (16.4% CP). There was a treatment x day effect for DMI ($P = 0.0005$).

* = $P < 0.05$ between treatments.

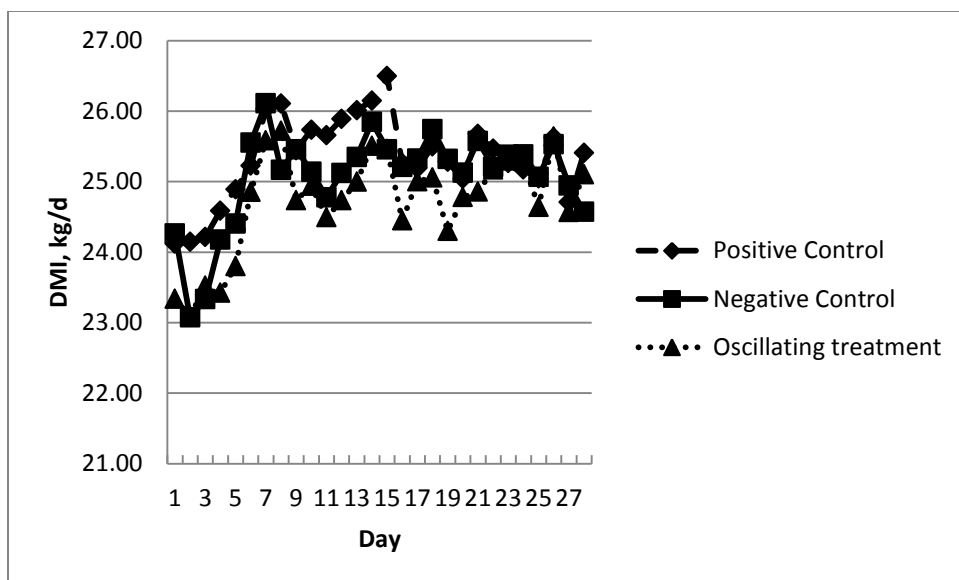


Figure 9: Average DMI over 28-d period. Each data point represents the average DMI of all 21 cows per treatment on each day of the period. DMI is variable no matter the treatment.

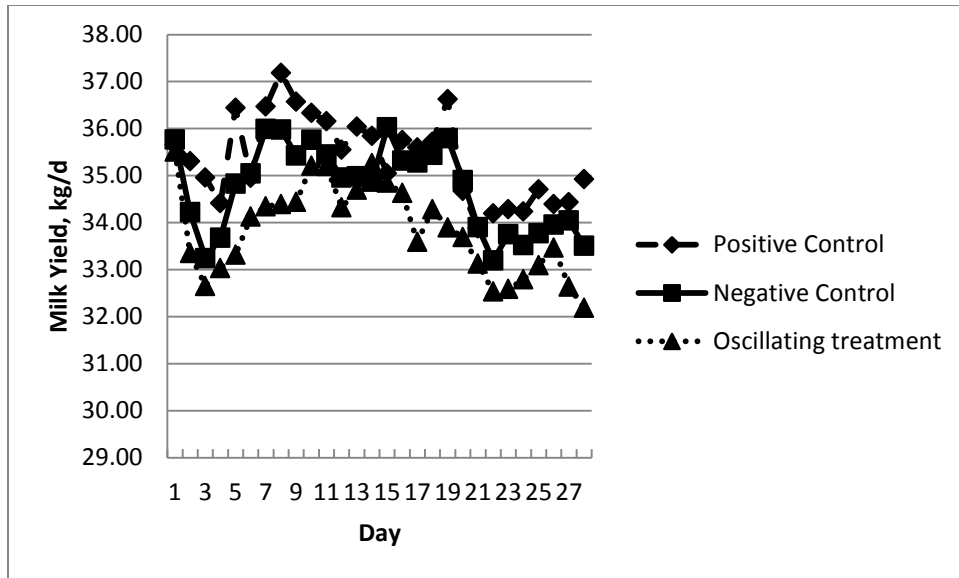


Figure 10: Average milk yield over 28-d period. Each data point represents the average milk yield of all 21 cows per treatment on each day of the period. For all treatments, milk yield decreased on average about 3 kg/d over the 28 d of a period.

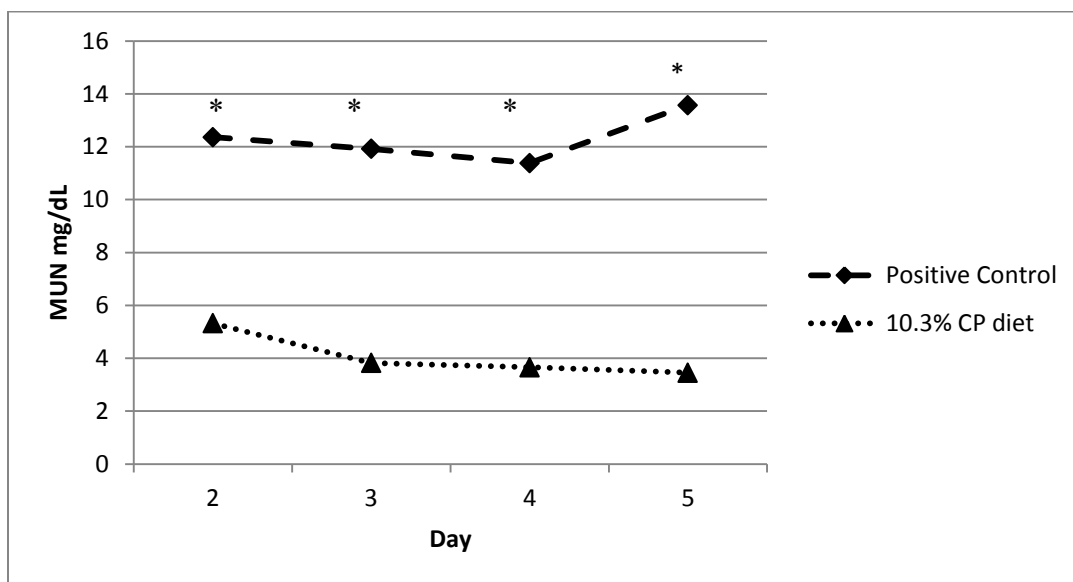


Figure 11: MUN for cows on the Positive Control versus cows on the 10.3% CP diet for 5 d. MUN for cows on the 10.3% CP diet was significantly lower ($P < 0.0001$) than MUN for cows on the Positive Control (16.4% CP) starting on d 2 (actually 1.5 days after cows were fed 10.3% CP diet since the diets changed after the AM milking on day 1).

* = $P < 0.05$ between treatments.

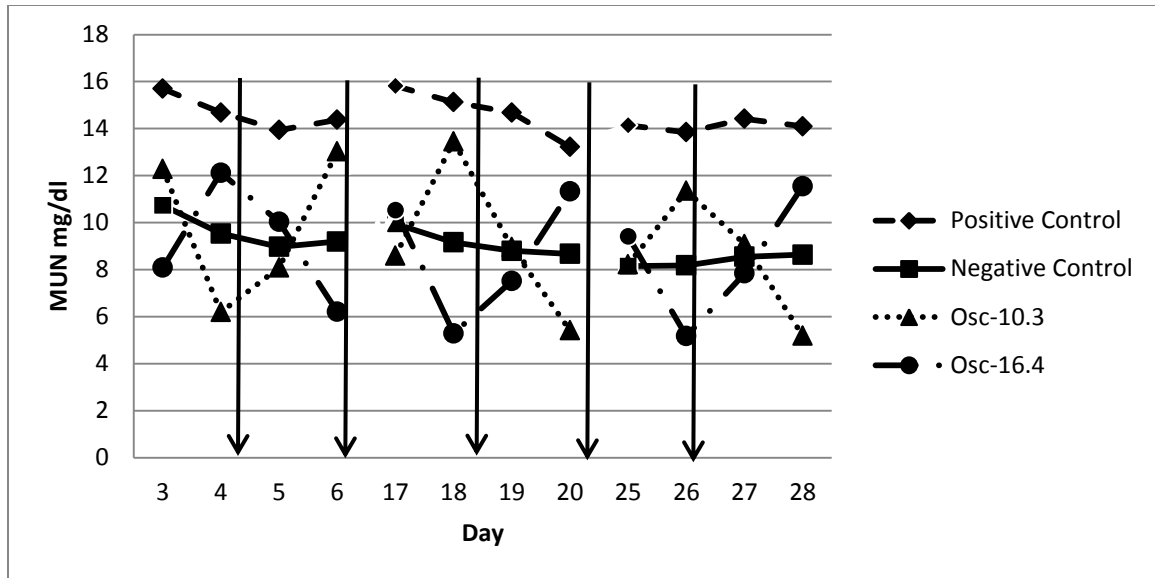


Figure 12: Treatment average daily MUN over the period. Arrows represent when feed was switched for the oscillating cows. MUN for cows ($n = 21$) on the Positive Control (16.4% CP) is higher ($P < 0.0001$) than those on the Negative Control ($N = 21$; 13.4% CP). On average, MUN for cows on Oscillating treatment (fed alternating 2 d Positive Control and 2 d 10.3% CP diet) is similar ($P = 0.38$) to those cows fed the Negative Control except that the MUN for oscillating cows follows a cyclic pattern over the period. MUN for both cows that started on the 10.3 % CP diet ($N = 11$; Osc-10.3) and oscillating cows that started on the positive control ($n = 10$; Osc-16.4) followed the cyclic pattern.

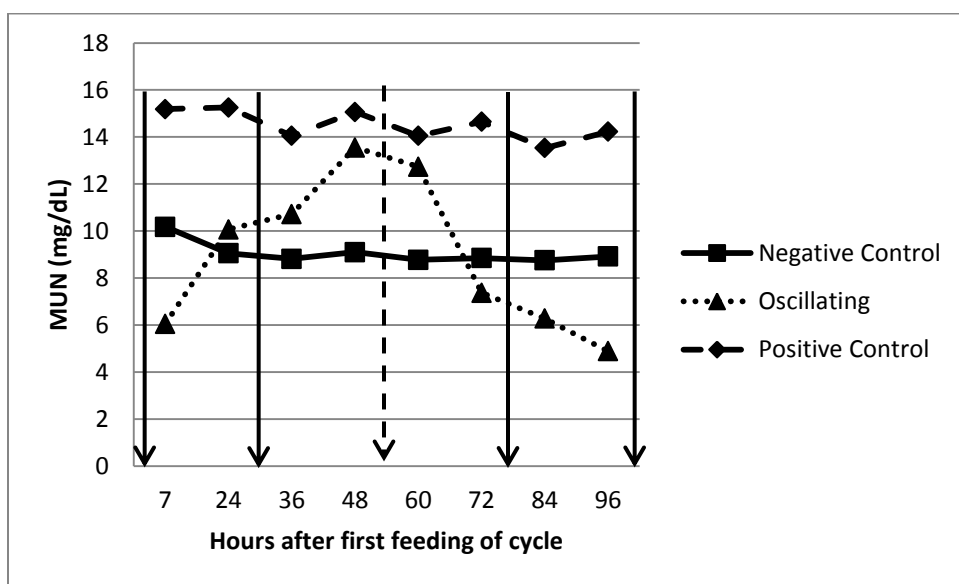


Figure 13: Average MUN over 8 milkings in one full oscillation cycle. The arrows indicate when cows were fed; the dashed arrow represents when feed was switched for the cows on the Oscillating treatment. Within 15 hours MUN increased by about 40% when Oscillating cows were switched to the Positive Control, and average MUN dropped by almost 70% within 12 hours after Oscillating cows were fed the Oscillating-10.3 diet. However, cows on the Oscillating treatment fed the Positive Control never had as high ($P = 0.01$) of MUN as those fed on the constant Positive Control treatment.

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